Therapeutic properties of hydrogen sulfide in ischemia / reperfusion injury

Bos, Eelke Marijn

THERAPEUTIC PROPERTIES OF HYDROGEN SULFIDE IN ISCHEMIA / REPERFUSION INJURY

Eelke M. Bos

THERAPEUTIC PROPERTIES OF HYDROGEN SULFIDE IN ISCHEMIA / REPERFUSION INJURY

Eelke Marijn Bos

Stellingen behorende bij het proefschrift:

THERAPEUTIC PROPERTIES OF HYDROGEN SULFIDE IN ISCHEMIA / REPERFUSION INJURY

Eelke M. Bos

1. Inductie van een hypometabole staat met waterstofsulfide (H_2S) beschermt organen tegen een periode van zuurstofgebrek (dit proefschrift).
2. Waterstofsulfide (H_2S) heeft protectieve effecten bij ischemie, ook als er geen hypometabolisme wordt bereikt (dit proefschrift).
3. Geïsoleerde organen kunnen met waterstofsulfide (H_2S) in een staat van laag metabolisme worden gebracht (dit proefschrift).
4. Cystathionine γ–lyase (CSE) is een endogene modulator van oxidatieve stress (dit proefschrift).
5. De hoeveelheid cystathionine γ–lyase (CSE) in een orgaan is van invloed op de uitkomst van transplantatie (dit proefschrift).
6. Er bestaat een hogere macht die meekijkt met alles wat we doen: de inlichtingendienst.
7. Het is merkwaardig dat er een houdbaarheidsdatum te vinden is op schimmelkaas.
8. De grootste tragedie in de wetenschap is de slachting van een prachtige hypothese door een lelijk feit (naar Thomas H. Huxley).
9. Een paar maanden in het laboratorium kunnen een paar uur zoeken in de wetenschappelijke literatuur voorkomen (naar Frank H. Westheimer).
10. Je kunt 100 jaar oud worden als je alle dingen opgeeft waarvoor je 100 jaar zou willen worden (naar Woody Allen).
11. Schrijf een pakkende stelling en je naam zal altijd voortleven (onbekend).

Eelke M. Bos
PhD-thesis

This PhD-project was financially supported by:
University Medical Center Groningen
Junior Scientific Masterclass, Faculty of Medicine, University of Groningen
Dutch Kidney Foundation
Research Institute GUIDE
Jan Kornelis de Cock foundation
Van Walree Fund, Royal Dutch Academy of Sciences
Carburos Metálicos, S.A.

The printing of this thesis was kindly supported by:
Astellas Pharma B.V.

Cover: Eelke M. Bos

Lay-out: Off Page

Printed by: Off Page

ISBN (Printed): 978-90-367-6421-6

ISBN (Digital): 978-90-367-6420-9

CMB.

RIJKSUNIVERSITEIT GRONINGEN

THERAPEUTIC PROPERTIES OF HYDROGEN SULFIDE IN ISCHEMIA / REPERFUSION INJURY

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
maandag 30 September 2013
om 16:15

door

Eelke Marijn Bos
geboren op 19 mei 1983
te Amsterdam

Promotores	Prof. Dr. H. van Goor
	Prof. Dr. R.J. Ploeg
Copromotor	Dr. H.G.D. Leuvenink
Beoordelingscommissie	Prof. Dr. R. Wang
	Prof. Dr. R. Goldschmeding
	Prof. Dr. H. Moshage

Paranimfen	W.B.W.H. Melenhorst
	G.C.G. Hugenholtz
	R.D. Zevenbergen

TABLE OF CONTENTS

Introduction		9
Chapter 1	Hydrogen sulfide – physiological properties and therapeutic potential in ischemia	17
Chapter 2	Hydrogen sulfide-induced hypometabolism prevents renal ischemia / reperfusion injury in mice	45
Chapter 3	Beneficial effects of gaseous hydrogen sulfide in hepatic ischemia / reperfusion injury	67
Chapter 4	Gaseous Hydrogen Sulfide protects against myocardial ischemia / reperfusion injury in mice partially independent of a hypometabolic state	91
Chapter 5	Cystathionine γ-lyase protects against renal ischemia / reperfusion by modulating oxidative stress	111
Chapter 6	Hydrogen sulfide-producing enzymes in pregnancy and preeclampsia	137
Chapter 7	Perspectives on donor organ preconditioning in heart beating renal transplantation	149
Chapter 8	Summary, discussion and future perspectives	169
	Nederlandse samenvatting	177
	List of abbreviations	179
	Author affiliations	183
	Dankwoord / Acknowledgements	185
	Publications	191

INTRODUCTION

Hydrogen sulfide (H_2S) has historically been perceived as a highly toxic and foul smelling molecule (Table 1). Since the recent discovery of H_2S as a physiological molecule produced in most cells of the mammalian body[1,2], the perceived image of H_2S has positively transformed. The amount of research performed on the subject has exploded in recent years, and has not shown signs of slowing down (Figure 1 and Figure 2). Over the past years, many new functions of H_2S in (patho)-physiology have been discovered, and hope for therapeutic applications of this substance has grown, with several companies established that exclusively develop H_2S-based therapeutics. However, the physicochemical properties of the molecule and the small therapeutic breadth/window hamper the translation to clinical applications. In addition, the former image of toxicity has not been erased, and bids for great caution in application of H_2S. The physiology and antioxidative effects of H_2S are extensively discussed in Chapter 1.

Table 1 - Overview of the known toxic effects of hydrogen sulfide.

Dose (parts per million)	Effect
0.02 ppm - 0.03 ppm	Detectable odor
5 ppm - 10 ppm	Offensive, unpleasant odor
20 ppm	Maximum allowable concentration (MAC) for daily 8 hour exposure
50 ppm	Irritation of the ocular conjunctiva
100 ppm	Loss of smell / olfactory nerve paralysis
100 ppm - 200 ppm	Upper respiratory tract irritation
300 ppm - 500 ppm	Pulmonary edema
500 ppm	Headache, dizziness, unconsciousness after 30 - 60 minutes of exposure
500 ppm - 700 ppm	Unconsciousness, respiratory paralysis eventually leading to death
1000 ppm	Rapid collapse, death within minutes

Adapted from Beauchamp et al.[3]

Toxicity of H_2S

The main focus of H_2S research before the 1990's was it's toxicity, stemming from accidents in the industrial setting such as oil and gas refineries, and in the farming industry. Accidents involving high concentrations of H_2S cause collapse, unconsciousness and respiratory paralysis, ultimately leading to death[3]. An overview of the effects of different concentrations of H_2S on human subjects is given above in Table 1.

Because of this toxic image, the use of H_2S – even in laboratory setting – is carefully monitored because of safety concerns. However, measurement of H_2S in the gaseous state is quite developed and reliable, and devices for ambient H_2S monitoring can ensure the safety of the patients and the medical personnel involved. We therefore consider gaseous H_2S or soluble forms of H_2S to be safe for application in the clinical setting.

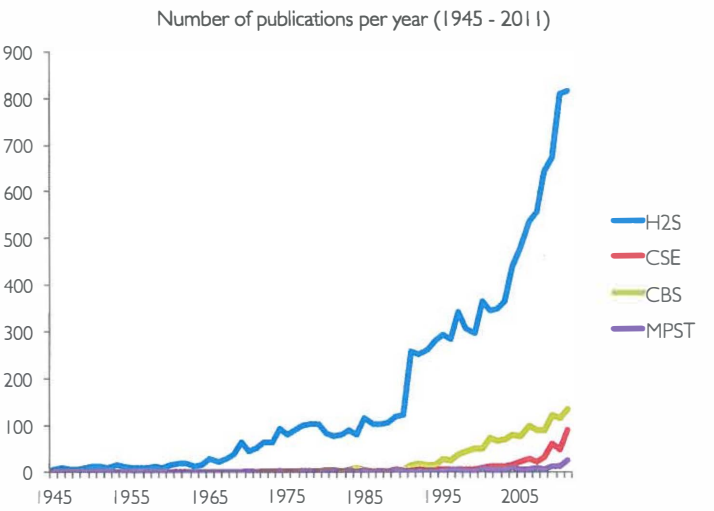


Figure 1 – Number of publications per year on H_2S, CSE, CBS and MPST from 1945 to 2011.
Data extracted from Web of Science.

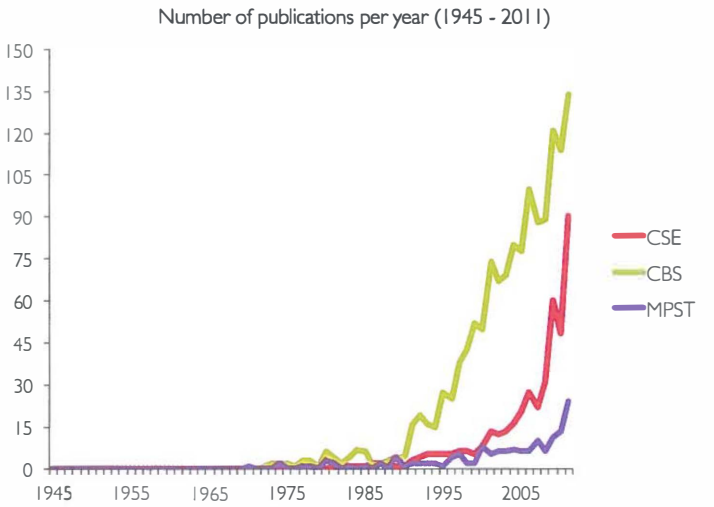


Figure 2 – Number of publications on the H_2S-producing enzymes CSE, CBS and MPST per year from 1945 to 2011.
Data extracted from Web of Science.

Endogenous production of H_2S

In recent years, the role and functioning of H_2S in physiology has become recognized. It is produced by three distinct enzymes in mammalian cells, namely cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MPST), and it

functions as a gaseous signaling molecule, or gasotransmitter, similar to nitric oxide (NO) and carbon monoxide (CO). Its functions range from vasodilatory action as an endothelial derived hyperpolarizing factor (EDHF) to modulation of oxidative stress and signaling through posttranslational protein modification[4-7]. Although many of the effects of H_2S have not been elucidated yet, it appears clear that H_2S is an important mediator in cellular physiology.

H_2S-induced hypometabolism

The current project was shaped following the discovery that gaseous H_2S could induce a reversible, hibernation-like state in mice, which was dubbed suspended animation[8]. During H_2S treatment, oxygen consumption and carbon dioxide production of these animals is reduced by 90-95%. Over time, core body temperature decreases to around 2°C above the ambient temperature. This massive reduction in oxidative metabolism is reversible after cessation of the H_2S exposure, without apparent toxic effects. This state of hypometabolism could bring great protection during states of low oxygen supply, such as ischemia. We were interested in protecting organs from ischemic damage, and we pursued H_2S as a promising candidate to achieve this.

The mechanism behind H_2S-induced hypometabolism has not been elucidated until now. One of the main hypotheses is the reversible inhibition of cytochrome c oxidase (COX), the terminal enzyme in the mitochondrial electron transport chain[9]. H_2S can non-competitively and reversibly bind to COX at oxidized sites, inhibiting the oxidative capacity of the electron transport chain[10]. This causes mitochondrial depolarization and ATP depletion[11]. In addition, H_2S can be used as an electron donor in the electron transport chain during depletion of O_2, which might be a mechanism for keeping only essential metabolic activity during hypoxia[12].

In our view, the application of hypometabolism is primarily applicable in ischemia with predictable onset, since our data indicate that the hypometabolic state is more potent when induced before or at the time of hypoxia, and not afterwards. This made organ transplant models the most rational for testing our hypothesis. It is highly interesting to see whether hypometabolism could protect in models of myocardial infarction or stroke, but the clinical relevance and treatment effect is likely smaller, since delivering H_2S to the affected area through the circulation is not possible during the ischemic event.

Organ donation and ischemia / reperfusion injury

One of the main determinants of outcome after solid organ transplantation is the amount of ischemia that the organ is exposed to[13,14]. The principal method for reducing the unfavorable effects of transplant anoxia is cooling the organ after removal from the donor. Cold ischemia greatly lengthens the time available between explantation and implantation in the donor. The effect of cooling is based on reducing metabolism and enzymatic activity in the organ. Cold storage does have detrimental effects as well, and some of those could be prevented by inducing hypometabolism using H_2S, or maybe cooling the organ to room temperature while adding H_2S to the preservation solution. Recent developments in machine perfusion of organs during preservation have improved outcome after renal transplantation. These developments also offer an opportunity for addition of H_2S to the perfusion solution to protect the transplant.

AIM OF THIS THESIS

The aim of this thesis was to investigate the therapeutic potential of H_2S in the setting of ischemic and hypoxic disease.

In Chapter 1 an outline of the known physiological functions of H_2S is given, complemented with the current state of research on H_2S in hypoxic models, and the developments that have occurred in recent years in the fields of H_2S delivery and measurement. In Chapter 2, Chapter 3 and Chapter 4 we test our hypothesis that H_2S-induced hypometabolism in protective in ischemic models of the kidney, liver and heart. Since the therapeutic window may vary between various organs and cell types, each chapter has its distinctive approach in regard to the timing, time course and dose of H_2S. Chapter 5 investigates the antioxidant role of endogenous H_2S production by CSE in ischemic settings in vitro, in vivo and in human renal transplant tissue, using models of CSE deficiency and overexpression. In chapter 6 we studied whether the expression of CSE and CBS, and thereby the possible endogenous production of H_2S, is altered in preeclampsia, a vascular disease associated with placental hypoxia and endothelial dysfunction. Chapter 7 recapitulates the effects of brain death on transplanted organs, and the opportunities it presents for protecting donor organs from transplant-related ischemia. In Chapter 8, all results are summarized and discussed, followed by a view on the future possibilities of H_2S related research and therapeutic applications.

REFERENCES

1. Szabó, C. Hydrogen sulphide and its therapeutic potential. *Nat Rev Drug Discov* 6, 917–935 (2007).
2. Wang, R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol. Rev.* 92, 791–896 (2012).
3. Beauchamp, R. O., Bus, J. S., Popp, J. A., Boreiko, C. J. & Andjelkovich, D. A. A critical review of the literature on hydrogen sulfide toxicity. *Crit Rev Toxicol* 13, 25–97 (1984).
4. Mustafa, A. K. *et al.* Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. *Circ. Res.* 109, 1259–1268 (2011).
5. Yang, G. *et al.* H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322, 587–590 (2008).
6. Bos, E. M. *et al.* Cystathionine γ-Lyase Protects against Renal Ischemia/Reperfusion by Modulating Oxidative Stress. *J Am Soc Nephrol* 24, 759–770 (2013).
7. Mustafa, A. K. *et al.* H2S signals through protein S-sulfhydration. *Sci Signal* 2, ra72 (2009).
8. Blackstone, E., Morrison, M. & Roth, M. B. H2S induces a suspended animation-like state in mice. *Science* 308, 518 (2005).
9. Dorman, D. C. *et al.* Cytochrome oxidase inhibition induced by acute hydrogen sulfide inhalation: correlation with tissue sulfide concentrations in the rat brain, liver, lung, and nasal epithelium. *Toxicol Sci* 65, 18–25 (2001).
10. Cooper, C. E. & Brown, G. C. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide. *J Bioenerg Biomembr* 40, 533–539 (2008).
11. Bos, E. M. *et al.* Hydrogen sulfide-induced hypometabolism prevents renal ischemia/reperfusion injury. *J Am Soc Nephrol* 20, 1901–1905 (2009).
12. Goubern, M., Andriamihaja, M., Nübel, T., Blachier, F. & Bouillaud, F. Sulfide, the first inorganic substrate for human cells. *FASEB J* 21, 1699–1706 (2007).
13. Kayler, L. K., Srinivas, T. R. & Schold, J. D. Influence of CIT-induced DGF on kidney transplant outcomes. *Am J Transplant* 11, 2657–2664 (2011).
14. Doshi, M. D., Garg, N., Reese, P. P. & Parikh, C. R. Recipient risk factors associated with delayed graft function: a paired kidney analysis. *Transplantation* 91, 666–671 (2011).

1

HYDROGEN SULFIDE – PHYSIOLOGICAL PROPERTIES AND THERAPEUTIC POTENTIAL IN ISCHEMIA

EM Bos
H van Goor
HGD Leuvenink

Submitted

ENDOGENOUS PRODUCTION AND FUNCTION

Hydrogen sulfide (H_2S) is now considered to be the third gaseous signaling molecule - or gasotransmitter - together with nitric oxide (NO) and carbon monoxide (CO). It is produced from L-cysteine by cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), and from 3-mercaptopyruvate by 3-mercaptopyruvate sulfurtransferase (MPST) (Figure 1). The interest in the field of H_2S research has grown markedly in recent years, with greatly reduced focus on toxicity and increased attention for the many physiological functions of endogenously produced and exogenously administrated H_2S.

Physiological function

The most widely studied function of endogenous H_2S relates to its vasodilatory effects, of which the mechanism has now been partially clarified. The functional properties of H_2S in the vascular bed can best be studied in genetically manipulated mice, such as the $CSE^{-/-}$ mice that has been developed in recent years, which need dietary cysteine to survive[1,2]. CSE deficiency causes hypertension, as evidenced by $CSE^{-/-}$ mice which show an increase in systolic blood pressure of ~18 mmHg[3]. This increase in blood pressure is similar to the effects seen in endothelial nitric oxide synthase (eNOS) knockout mice[4]. $CSE^{-/-}$ mice have diminished endothelium dependent vasorelaxation which is likely related to the vasorelaxant effects of H_2S. These effects are mediated through the opening of ATP-dependent K^+ channels (K_{ATP}) in vascular smooth muscle cells[5]. Recently it has been shown that the mechanism behind the opening of K_{ATP} channels is the direct sulfhydration of the Kir6.1 subunit of this channel by H_2S, and this effect is absent in $CSE^{-/-}$ mice, indicating an essential role for CSE/H_2S in this vasodilatation[6]. CBS regulates the cerebral microcirculation and CBS deficient mice show reduced or absent vasodilation in precapillary arterioles in response to hypoxia, while CSE deficiency has no effect[7].

CSE and CBS both are involved in the response to oxidative stress. $CSE^{-/-}$ animals are more susceptible to ischemic damage[8], and overexpression of CSE in cardiac tissue protects from myocardial infarction[9]. This seems to be related to the reduction of destructive reactive oxygen species (ROS) by H_2S[8,10] and the protection of mitochondrial integrity and function[9]. Mice deficient in CBS show increased amounts of oxidatively modified proteins in their livers[11], indicating a role for H_2S in the antioxidative response. CSE and CBS can be excreted by endothelial cells and hepatocytes, circulate in plasma and actively produce H_2S from homocysteine in human blood[12] and this extracellular CSE and CBS protect endothelial cells from redox stress.

H_2S is essential for vascular endothelial growth factor (VEGF) mediated angiogenesis. Exogenous H_2S increases endothelial cell proliferation and migration, and deficiency of CSE causes impaired microvessel formation in aortic rings[13]. In $CSE^{-/-}$ mice, wound healing is impaired and treatment of rats with NaHS improves wound healing[13,14]. These beneficial effects were related to modulation of angiogenic mechanisms. The actions of endogenous H_2S seems to be dependent on NO, and might act through a cyclic guanosine 5'-monophosphate (cGMP) mediated mechanism[14]. In addition, CSE seems to be involved in models of diabetes[15], possibly through its regulating role in the function of pancreatic β-cells[16,17].

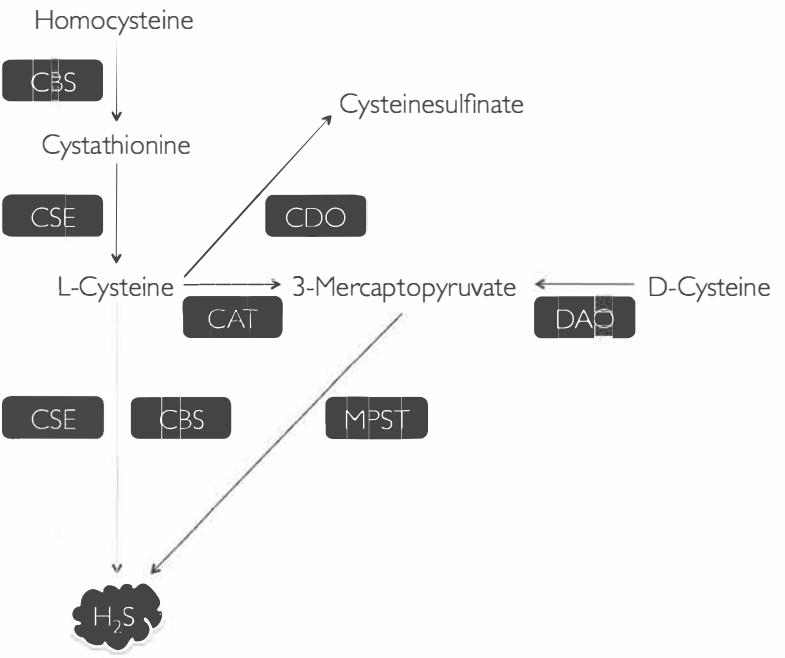


Figure 1 – Overview of endogenous H_2S production and the enzymes involved.
Abbreviations: CSE – cystathionine γ-lyase; CBS – cystathionine β-synthase; MPST – 3-mercaptopyruvate sulfurtransferase; CAT – cysteine aminotransferase; CDO – cysteine dioxygenase; DAO - D-amino acid oxidase.

Deficiency of CBS can cause vascular and endothelial dysfunction and cerebral interstitial remodeling[18,19], and is associated with increased oxidative stress[11]. Lack of CBS produces hyperhomocysteinemia, in humans as well as in mice. The $CBS^{+/-}$ mouse has been extensively used as a model for this disease. $CBS^{-/-}$ animals die at a very young age (3-4 weeks after birth), and are not frequently used for research purposes[20]. In Drosophila, CBS is necessary for the increased lifespan linked to dietary restriction[21]. CBS is encoded on chromosome 21, and patients with Down's syndrome have triple the amount of CBS protein in their brain[22]. It is still a topic of discussion whether this is related to the reduced mental capacity in this syndrome. MPST is the least studied of the H_2S producing enzymes, and has been mostly associated with the central nervous system – with activity in the brain and retina – and the vascular endothelium[23-25]. Intramitochondrial MPST has been shown to produce H_2S, where it is able to maintain energy production by donating electrons[26,27].

H_2S can be used as an inorganic energy substrate for mitochondria[27]. Under stress, such as increased intracellular calcium concentrations, CSE can translocate to the mitochondria mediated by translocase of the outer membrane 20 (Tom20)[28]. It is hypothesized that translocation of CSE to the mitochondria can increase mitochondrial H_2S levels to be used as an electron donor in the electron transport chain.

Regulation of H_2S

CSE and CBS expression are regulated through the specificity protein 1 (Sp1) transcription factor[17,29-31]. Recent research suggests that microRNA 21 (miR21) reduces the expression of Sp1 and CSE by directly targeting Sp1[32]. ERK mediated phosphorylation of Sp1 reduces its activity[30]. CSE expression through Sp1 can also be activated by TNFα[33]. Nrf2, a transcription factor that is activated by oxidative stress, can translocate the nucleus upon activation and bind to an antioxidant responsive element (ARE) to mediate gene transcription of protective genes such as glutathionine S-transferase (GST) or heme oxygenase 1 (HO-1)[34]. The CSE promotor contains an ARE, and thus the expression of CSE can be modulated by Nrf2[35]. Modulation of Sp1 or Nrf2 might be a strategy to affect CSE expression and endogenous H_2S production. Also, the CSE promotor contains an farnesoid X receptor (FXR) responsive element, activation of which induces expression of CSE and mutation blocks FXR mediated CSE expression[36]. It has been shown that CSE and CBS can be posttranslationally modified by sumoylation. The activity of CBS is decreased by sumoylation, and it can theoretically be involved in nuclear translocation, although this has not been directly shown for CBS or CSE[37].

Recently, a new pathway for the production of H_2S from D-Cysteine has been uncovered, mainly by MPST in the cerebellum and kidney[38], involving the activity of D-amino acid oxidase (DAO). Cysteine aminotransferase (CAT) produces 3-mercaptopyruvate from cysteine, and could be involved in the regulation of H_2S production[39]. Thioredoxin or dihydrolipoic acid (DHLA) are necessary for production of H_2S from MPST[40]. CAT activity in retinal neurons is modulated by Ca^{2+}[39]. Cysteine dioxygenase (CDO) is responsible for the majority of cysteine catabolism. In models of CDO deficiency, the increase in cysteine metabolism causes excess H_2S production through CSE and CBS, and related toxicity[41,42]. For an overview, see Figure 1.

Ethylmalonic encephalopathy protein 1 (Ethe1) has recently been implicated in the catabolism of sulfur compounds, as deficiency of Ethe1 causes sulfide toxicity in mice[43]. Interestingly, tissue specific knockout of Ethe1 in muscle, liver or brain caused localized signs of sulfide toxicity without increasing urinary thiosulfate, indicating that local levels as opposed to serum levels of H_2S are important[44], which could be due to the high volatility and reactivity of sulfide. These findings have already led to a promising therapy for ethylmalonic encephalopathy in humans using metronidazole to reduce intestinal H_2S production by bacterial flora and N-acetylcysteine to neutralize H_2S[45].

One of the most interesting properties of H_2S is the sulfhydration of proteins and modifying their activity, as touched upon before. This posttranslational protein modification, where an SH-group is added to a reactive cysteine residue, can change the activity of the target protein[46]. This mechanism is analogous to protein phosphorylation. It was first shown that 10%-25% of the abundant GAPDH protein was sulfhydrated in vivo, and that sulfhydrated GAPDH had higher activity. Since then, well studied proteins such as NF-κB, VEGFR2, PTP1B, H-Ras, the K_{ATP}-channel and Keap1 were shown to be modified by H_2S, with associated changes in protein activity[6,33,47-51]. This is a field still in its infancy, but it seems that it can become a meaningful aspect of intracellular signaling pathways.

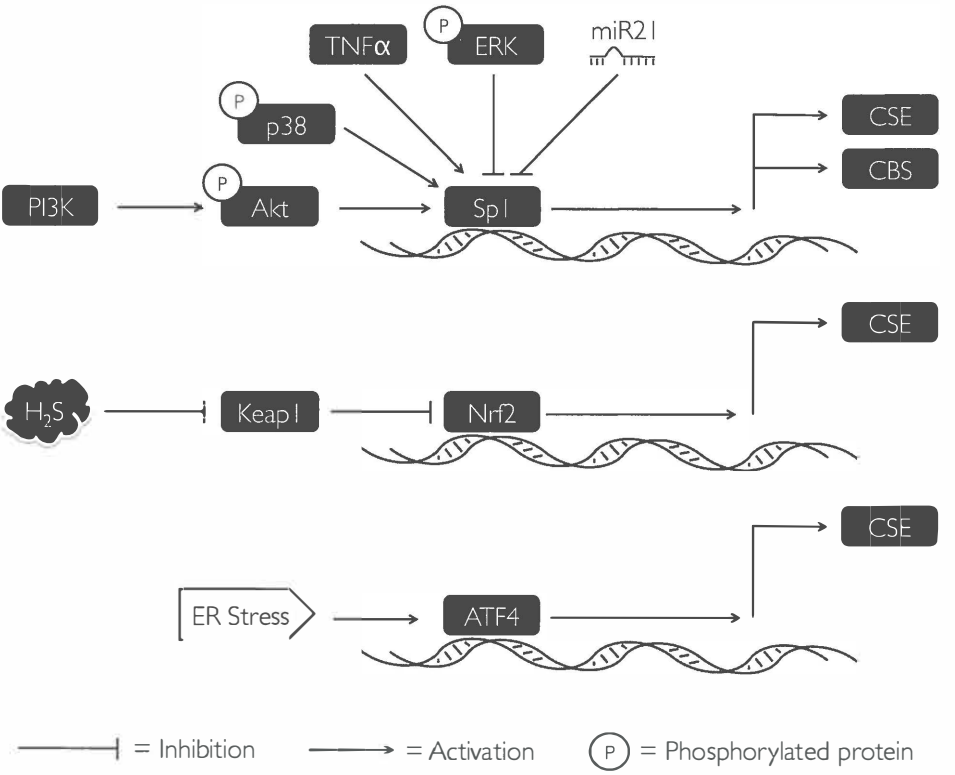


Figure 2 – Overview of endogenous CSE and CBS regulation.
Abbreviations: CSE – cystathionine γ-lyase; CBS – cystathionine β-synthase; ERK – Extracellular signal related kinase; TNFα – tumor necrosis factor alpha; PI3K – phosphoinositide 3 kinase; Akt – protein kinase B; Sp1 – specificity protein 1; Keap1 – kelch-like ECH-associated protein 1; Nrf2 – nuclear factor (erythroid-derived 2)-like 2; ATF4 – Activating transcription factor 4; ER – endoplasmatic reticulum.

EXOGENOUS ADMINISTRATION OF H_2S

There are currently several options for altering H_2S levels in experimental settings:

- Sulfide-sodium salts (NaHS, Na_2S)
- Exposure to gaseous H_2S
- Slow-releasing H_2S donors
- Hybrids of H_2S-donors and known substances
- Cysteine analogues
- Modulating the expression or activity of H_2S-producing enzymes

The physicochemical properties of H_2S cause several problems for the application in biomedical experiments. In the large majority of the published literature, one of the sodium salts that release H_2S in solution have been used (NaHS or Na_2S). The major problem of using sulfide salts as H_2S donors is the very quick loss of sulfide from solution and the rapid reactivity and catabolism of the H_2S[52-55].

Over the course of years, it has become clear that these sodium salts have disadvantages, such as the rapid peak and concurrent decrease in H_2S levels when dissolved or injected[53,56]. This makes it difficult to achieve controlled, stable and therapeutic levels in vitro and in vivo.

The development of slow-releasing H_2S donors has improved the ability to achieve such a stable increase in H_2S levels[52-54,56,57]. Addition of NaHS or Na_2S to a pH-neutral solution leads to an increase in HS^- and H_2S levels, with the peak level occurring within minutes, after which the H_2S rapidly dissolves from solution, or quickly reacts with proteins so that the amount of H_2S decreases to normal levels in 30 minutes to 3 hours[52-54]. Although some postulate that one of the two main sulfide-sodium salts is superior to the other, the little comparative work that has been performed shows no difference between the compounds[58].

The exposure of animals or cells to gaseous H_2S poses some technical problems that mainly revolve around the toxicity and related safety measures that need to be taken when using pressurized H_2S-containing gases. In addition, the corrosive nature of H_2S prompts the need for specialized materials. The use of gaseous H_2S can induce very stable states of hypometabolism in mice[59,60]. Gaseous H_2S can be used for small- and large animal experiments or for the exposure of cells or nematodes[59,61,62]. The actual concentration that is concurrently delivered with different concentrations of gaseous H_2S to target organs is however still a point that has not been elucidated.

In recent years, two major developments in this field have occurred. First, compounds that slowly release H_2S have been produced (see Table 1). These have made it possible to study the effects of long term heightened H_2S levels in vitro and in vivo. This as opposed to the large and short peaks in H_2S levels that are produced by the sulfide releasing sodium salts, where by daily injection the serum H_2S levels are most likely very high for a short period and near normal for the majority of the day.

The second development is the creation of hybrids from known and widely used drugs with sulfide releasing compounds. These new conjugates can potentiate the effects of the original, or diminish the side effects of the old drug. A large proportion of the hybrids currently under development are NSAIDs. For example, ACS14 (S-aspirin), a H_2S releasing form of aspirin, has a broader inhibitory effect on platelet aggregation compared to aspirin, showing effect on ADP- and thrombin receptor activating peptide (TRAP)-induced aggregation where aspirin has minimal effect.[63] ATB-346, a H_2S releasing form of naproxen, greatly reduces the gastrointestinal mucosal damage associated with NSAID usage[64,65]. ACS15 (S-diclofenac) inhibits angiogenesis, has strong anti-inflammatory effects in LPS- or Amyloidβ-induced inflammation and attenuated the detrimental effects of ischemic myocardial damage in rats[52,66-68]. Other examples are ACS6 (S-sildenafil), which has smooth muscle relaxant effects on the corpus cavernosum, but also showed antioxidative effects in endothelial cells[69,70]. ACS83 (S-Levodopa) increased dopamine and GSH levels in the brain and reduced the glial inflammatory response[71].

An alternative development is the use of naturally occurring cysteine analogues such as diallyl trisulfide (DATS, can be derived from garlic[72]) or S-propargyl cysteine (SPRC). These compounds seem to increase H_2S levels for longer periods of time and have protective effects in models of ischemia[54,73,74].

Another option for H_2S based treatments is modulation of the expression or activity of H_2S producing enzymes. There are several compounds that inhibit the activity of CSE or CBS.

Table 1 - H_2S Donors - Overview.

Name	Substance	Producer	Effects	Reference
Non-steroidal anti-inflammatory drugs (NSAID) conjugates				
ACS14	H_2S-releasing aspirin	CTG Pharma	Increased H_2S and GSH levels in rats. Reduced glial cell inflammatory response to LPS and TNFα. Reduced thrombus formation, broader inhibition of platelet aggregation versus aspirin. Prevented atherosclerotic plaque formation in apoE$^{-/-}$ mice Reduced hemodynamic effects of GSH depletion, reduces gastric damage. Prevented gastrointestinal damage, increases GSH levels, induces HO-1. Reduced cell death and ROS in retinal neurons and increases GSH levels.	Guistarini (2010) Lee - Glia (2010) Pircher (2012) Zhang (2012) Rossoni (2010) Sparatore (2009) Osborne (2012)
ACS15	H_2S-releasing diclofenac	CTG Pharma	Inhibited VSMC proliferation and induced apoptosis. Reduced pancreatitis associated lung injury. Reduced glial cell inflammatory response to LPS and TNFα. Reduced LPS induced inflammatory response in mice. Reduced Amyloidβ induced cell death and inflammation in microglia. Attenuated ischemic damage in isolated perfused hearts. Inhibited vascular outgrowth from tumor and muscle, reduced endothelial proliferation.	Baskar (2008) Bathia (2008) Lee - Glia (2010) Li (2007) Lui (2011) Rossoni (2008) Isenberg (2012)
ATB-346	H_2S-releasing naproxen	Antibe Therapeutics	Reduced NSAID associated gastrointestinal damage. Reduced arthritis induced hindpaw edema, reduced inflammation, no gastric damage. Inhibits L-NAME-induced hypertension.	Blackler (2012) Wallace (2010) Wallace (2010)
ATB-429	H_2S-releasing mesalamine	Antibe Therapeutics	Improved antinociceptive activity in rat model of colorectal distension.	Distrutti
ACS18	H_2S-releasing sulindac	CTG Pharma	Inhibited vascular outgrowth from tumor and muscle, reduced endothelial proliferation.	Isenberg (2012)
ACS21	H_2S-releasing salicylic acid	CTG Pharma	Reduced hemodynamic effects of GSH depletion, reduced gastric damage. Did not cause gastrointestinal damage, increased GSH levels.	Rossoni (2010) Sparatore (2009)
NBS-1120	NO- and H_2S-releasing aspirin		Reduces proliferation and induces apoptosis in colon cancer cells, and reduces tumor volume in a mouse model of colon carcinoma.	Chattopadhyhay (2012)

Other conjugates				
ACS2 / 33 / 43	H_2S-releasing valproic acid	CTG Pharma	Increased histone deacetylase inhibition compared to valproic acid Inhibited vascular outgrowth from tumor and muscle, reduced endothelial proliferation.	Perrino (2008) Isenberg (2012)
ACS6	H_2S-releasing sildenafil	CTG Pharma	Antioxidative properties in arterial endothelial cells Similar effects to sildenafil on corpus cavernosum relaxation and reduces ROS. Attenuated Hcy-induced cell death, apoptosis and ROS.	Muzaffar (2008) Shukla (2009) Tang (2012)
ACS67	H_2S-releasing latanoprost	CTG Pharma	Reduced intraocular pressure in model of glaucoma, increases GSH levels. Protected from retinal ischemia, reduces apoptosis and ROS.	Perrino (2009) Osborne (2010)
ACS83 / 84 / 85 / 86	H_2S-releasing Levodopa	CTG Pharma	Increased dopamine and GSH levels in the brain. Improved cellular viability and reduced inflammatory response of glial cells to activation.	Lee - JBC (2010) Lee - JBC (2010)
S-memantine	H_2S-relasing memantine	CTG Pharma	Improved neurological outcome and smaller infarct volume in brain ischemia.	Marutani (2012)
Single H_2S donors				
NaHS	Sulfide-sodium salt	Generic	Releases H_2S very rapidly	
Na_2S	Sulfide-sodium salt	Generic	Releases H_2S very rapidly. Under development by Ikaria under the name IK-1001	
GYY4137	Slow releasing H_2S donor	Cayman Chemical	Reduced proliferation and cell survival in multiple cancer cell lines. Reduced in-vivo tumor growth of two leukemia cell lines. Attenuated hemodynamic and inflammatory response to LPS.	Lee (2011) Lee (2011) Li (2008)
SG-1002	Mixture, mainly Sulfide-ring	Sulfagenix	Reduced cardiac hypertrophy after transverse aortic constriction in WT and $CSE^{-/-}$ mice	Kondo (2013)
ADTOH	Dithiolthiene	Generic	Reduced hemodynamic effects of GSH depletion, reduced gastric damage. Increased GSH levels in plasma, aorta and heart.	Rossoni (2010) Sparatore (2009)
DATS	Diallyl trisulfide	Generic	Reduced ischemic damage in myocardial infarction, protected mitochondria.	Predmore (2012)
SPC	S-allyl-L-cysteine	Generic	Reduced oxidative stress in rat model of myocardial infarction.	Wang (2010)
SAC	S-propyl-L-cysteine	Generic	Reduced oxidative stress in rat model of myocardial infarction.	Wang (2010)
SPRC	S-propargyl-cysteine	Generic	Induced apoptosis and reduced tumor growth of gastric cancer cells in-vivo Reduced oxidative stress in rat model of myocardial infarction.	Ma (2011) Wang (2010)
NACET	N-acetyl-L-cysteine ethyl ester	Generic	Increased H_2S levels in plasma, is rapidly taken up by erytrocytes.	Guistarini (2012)
N-(benzoylthio) benzamide	Cysteine-activated H_2S donor	Generic	Increased H_2S levels only in the presence of L-cysteine.	Zhao (2011)

These are however not highly specific. Also, since the therapeutic effects of H_2S mostly are related to increased levels, use of these substances has been mostly shown to be detrimental in model systems. CSE inhibitors are DL-propargylglycine (PPG) and β-cyano-L-Alanine (BCA). CBS inhibitors are amino-oxyacetate (AOA) and hydroxylamine (HA). S-adenosyl-L-methionine can increase CBS activity in vivo[75]. As mentioned before, modulation of Sp1, Nrf2, FXR or miR21 might be targets for modulation of expression of CSE and CBS.

Loss of Ethe1, an endogenous dioxygenase, causes the disease ethylmalonic encephalopathy, and greatly increases H_2S levels in serum and organs of Ethe1$^{-/-}$ animals[43]. Modulation of the activity of Ethe1 might cause increased levels of H_2S in vivo. Another option is targeting cysteine dioxygenase (CDO), which is involved in cysteine catabolism, and CDO$^{-/-}$ and CDO$^{+/-}$ animals have increased tissue levels of sulfide[42] and show evidence of sulfide toxicity[41]. However, all options to increase endogenous H_2S production can lead to toxicity through depletion of cytochrome c oxidase (COX) and local organ damage, as shown in a conditional organ specific knockout model of Ethe1[44], where local Ethe1 deficiency caused increased levels of H_2S and associated confined toxicity in the affected organs.

Another interesting mechanism is intracellular translocation to modify protein function. CSE can be translocated to mitochondria, which might affect the function it exerts. This process is regulated by translocase of the outer membrane 20 (Tom20)[28]. When this process can be regulated it might lead to new therapies to modulate the function of CSE. Sumoylation of CBS and CSE has also been executed, where CBS decreases in activity when sumoylated, the effects on CSE have yet to be determined[37], and it remains to be determined what the localized effects are of the associated nuclear translocation.

THERAPEUTIC POTENTIAL OF H_2S IN OXIDATIVE STRESS

One of the most prominent and well studied properties of H_2S is the reduction in damage related to oxidative stress. Many groups have investigated the role of H_2S in ischemia/reperfusion injury (IRI) or in vitro oxidative stress, and as a consequence many different models and treatment regimens have been used. The mechanism behind H_2S induced protection has not been clearly elucidated, although many different approaches have been investigated. It seems that H_2S has many different effects and it is not possible to point to a single mechanism that explains all the effects in these models.

In vivo models of ischemia

Heart

Myocardial infarction (MI) is one of the main models used for the study of the protective effects of H_2S in ischemia. Intravenous Na_2S 24 hours before cardiac ischemia attenuated infarct size, serum troponin levels, lipid hydroperoxide levels and apoptosis, while cardiac function improved[76]. Injection of Na_2S in the left ventricular lumen at reperfusion attenuated myocardial infarct size, troponin levels, inflammation, apoptosis and cardiac function. Na_2S preserves mitochondrial oxygen consumption and -integrity after myocardial ischemia[9]. Other studies have shown similar effects of NaHS or Na_2S in models of MI, using different models, treatment

regimens and modes of administration[51,77-81]. In a mouse model with NaHS in drinking water for 4 weeks after MI, H_2S protected against cardiac function loss and fibrosis, and NaHS increased the amount of collaterals formed 4 weeks after MI[82]. Na_2S improved myocardial function after cardiac arrest and cardiopulmonary resuscitation (CA/CPR)[83]. Na_2S induced a significant improvement of the recovery of myocardial and endothelial function in a canine model of cardiopulmonary bypass with hypothermic cardiac arrest[84]. From our own experiments, we have seen cardioprotective effects of gaseous H_2S in hypometabolic as well as non-hypometabolic concentrations. Sub-hypometabolic concentrations of H_2S prior to ischemia protect hearts from ischemia-induced fibrosis and inflammation, whereas inducing hypometabolism offers additional protection against short term myocardial necrosis[85].

Not only treatment with exogenous H_2S seems to be protective, modulation of the endogenous production is a promising approach as well. Cardiac specific overexpression of CSE increases H_2S production rate[9] and improves survival and reduced ventricular dilatation and hypertrophy in a model of heart failure induced by permanent left coronary artery (LCA) occlusion. Daily administration of Na_2S attenuated oxidative stress and mitochondrial dysfunction during the development of ischemia-induced heart failure[86]. After MI, cardiac specific CSE overexpression reduced left ventricular dilatation and hypertrophy, and improved left ventricular function[9]. In rats NaHS reduced, while inhibition of CSE using PAG increased infarct size. PAG treated animals had increased inflammation after MI[87,88].

Kidney

In renal ischemia/reperfusion injury, NaHS at clamping and during reperfusion attenuated the rise in serum creatinine and improved microvascular flow. Necrosis and apoptosis and inflammation are attenuated by NaHS[89,90]. IRI associated liver injury is attenuated by NaHS after renal IRI[89]. In another study bilateral renal ischemia caused mortality, renal failure and inflammation, which were prevented by a gaseous H_2S-induced hypometabolic state (without hypothermia), but only when H_2S was given before ischemia (pretreatment)[62]. Structural damage and apoptosis caused by IRI was nearly abolished by pretreatment and combined pre/posttreatment, while posttreatment alone had less but significant effects[62]. Transmission electron microscopy showed that H_2S pre-/posttreatment prevented IRI-induced mitochondrial swelling and degeneration[62]. In a model of porcine aortic occlusion, renal function loss was attenuated by Na_2S, as were Oxidative DNA base damage, IL-6 and IL-1beta levels[91].

In the kidney, production of H_2S by CSE seems to modulate the effects of oxidative stress. In $CSE^{-/-}$ animals – which have low renal H_2S levels – mortality, renal function loss and DNA damage is increased following bilateral ischemia reperfusion injury. These effects could be rescued by NaHS treatment[8]. In human transplant biopsies, the level of CSE mRNA pre-transplantation was associated with improved outcome as measured by renal function after kidney transplantation[8]. After bilateral renal ischemia, CSE protein, renal H_2S production and plasma H_2S levels are increased. NaHS injections reduces IRI-induced renal function loss[92]. Bilateral IRI induced renal function loss was exacerbated by PPG treatment, while NaHS treatment attenuated these effects. NaHS also reduced acute tubular necrosis and apoptosis[93]. Interestingly, D-cysteine can

protect kidneys from ischemia as well, possibly with increased potency compared to L-cysteine probably by the production of H_2S through D-amino acid oxidase (DAO) and MPST[38].

Brain

In middle cerebral artery occlusion in rats, gaseous H_2S for 2 days starting after reperfusion improved neurological outcome, and reduced infarct size and inflammation[94]. NaHS injection increased performance on maze escape latency and protected against histopathological damage in the hippocampal area in bilateral common carotid occlusion[95]. In models of stroke, not all studies show unequivocal protective effects, however. In global cerebral ischemia in rats low dose NaHS protected neurons, while high dose NaHS exacerbated neuronal damage[96]. Another study showed that infarct volume was increased in animals treated with NaHS. Both CBS inhibitors (AOAA and HA) and CSE inhibitors (BCA and PPG) reduced cortical H_2S production, and all inhibitors reduced infarct volume 24 hours after ischemia (with the CBS inhibitors being more potent)[97].

In a mouse model of cardiac arrest and cardiopulmonary resuscitation (CA/CPR), Na_2S improved survival, neurological function and neuronal survival. Serum H_2O_2 levels were reduced by Na_2S shortly after CPR indicating antioxidative effects. Mice overexpressing CSE in cardiomyocytes had increased survival and neurological outcome after CA/CPR[83]. In another study, short term neurological outcome was better in the sulfide group, but was similar 1 week after CA/CPR. Here, apoptosis and neuronal death was not affected by Na_2S[98]. Interestingly, a gain of function polymorphism in CBS has recently been associated with less delayed cerebral ischemia following aneurysmal subarachnoid hemorrhage in humans[99].

Liver

In a model of total hepatic IRI in rats, posttreatment with NaHS reduced plasma ALT after 6 h of reperfusion. Hepatic IRI associated renal and cardiac damage was attenuated by NaHS, as were plasma levels of MDA[100]. Pretreatment with gaseous H_2S in of partial hepatic ischemia reduces necrosis, AST, ALT, apoptosis, granulocyte influx, IL-6, IL-1beta and TNFα upregulation and ROS production. HO-1 expression was not associated with protection[101]. In partial hepatic ischemia, low dose Na_2S treatment (0.3 and 1 mg/kg) protected from liver damage, while 2 mg/kg increased damage, as measured by AST and ALT. H_2S treatment preserved GSH stores and attenuated lipid hydroperoxide and Caspase3 cleavage[102]. Treatment with NaHS 30 minutes before and after reperfusion, AST and ALT levels were attenuated, while PAG treated animals had levels similar to control IRI animals. Histological damage was reduced by NaHS, and increased by PAG. Inflammation and lipid peroxidation were reduced by NaHS, but unaffected by PAG[103].

NaHS suppressed hepatic autophagy in vitro and in vivo, and pharmacologically reducing autophagy further diminished the protective effect of NaHS, while rapamycin reversed the autophagyinhibitory effect and enhanced the protective effect of NaHS against IRI[104]. Interestingly, $CBS^{-/-}$ mice have 30% increased oxidatively modified proteins in liver samples[11], indicating an antioxidative function of CBS and/or endogenous H_2S under physiological circumstances.

Shock

In hemorrhagic shock in rats, gaseous H_2S and intravenous Na_2S greatly increased survival[105] and injection of NaHS 10 minutes before reperfusion reduced superoxide levels in aorta and heart[106].

In a porcine model of hemorrhagic shock, Na_2S infusion improved survival compared to controls, and troponin levels were reduced in Na_2S treated animals. Renal function (plasma creatinine and creatinine clearance) was improved by pre-/posttreatment with Na_2S. IL-6 levels were reduced by pre-/posttreatment, and lung, liver and kidney histopathological scores were improved[107]. However, in another porcine study Na_2S had no effect on survival or organ injury parameters[108].

Fascinatingly, in full body hypoxia in mice (5% O_2), animals survived for a median time of 12 minutes and a maximum time of 17 minutes, while 20 minutes of gaseous H_2S pretreatment followed by 1 hour of 5% O_2 did not cause any mortality or morbidity[60], with one experiment showing survival for more than 6 hours.

Lung

In an ex vivo model of lung transplantation related IRI with cold storage in rabbits, NaHS was given during reperfusion. ROS levels were greatly decreased by NaHS treatment[109,110]. In cold storage and reperfusion of rabbit lungs treated with inhaled H_2S, on reperfusion, pretreatment with inhaled H_2S caused better oxygenation and ventilation and lower pulmonary artery pressure[110]. In isolated lung perfusion, H_2S treatment attenuated histological damage caused by IRI, while PPG increased damage, and H_2S normalized markers of oxidative stress[111].

Intestine

NaHS reduces oxidative stress and structural damage, while it increased intestinal GSH levels and SOD activity in a model of rat intestinal IR[112,113]. In a model of jejunal ischemia, NaHS treatment preserved villus height after reperfusion[114]. Leukocyte rolling and adhesion are attenuated by NaHS[115].

In vitro studies

In models of hypoxia, NaHS attenuated apoptosis in endothelial cells as well as fibroblasts[116], and reduced ROS in hippocampal neurons[117] and cardiomyocytes[118]. In two different cell types, $CoCl_2$ induced chemical hypoxia induced cell death, apoptosis and ROS, which was attenuated by NaHS[119,120]. NaHS protects neuroblastoma cells from oxygen-glucose deprivation (OGD) / $Na_2S_2O_4$ induced cell death[121]. In neuroblastoma cells, NaHS concentration-dependently reduced formation of nytrotyrosine caused by $ONOO^-$, and protected from cell death[122]. HOCl-induced protein oxidation, lipid peroxidation and loss of cell viability were concentration-dependently inhibited by NaHS[123]. Overexpression of CBS reduced $Na_2S_2O_4$-induced apoptosis[121]. NaHS protects primary rat astrocytes from H_2O_2-induced cell death and ROS production, while inhibition of CBS decreased viability[124]. Also, NaHS protected photoreceptor cells from light-induced degeneration, reducing apoptosis and oxidative stress[39]. Isolated mitochondria exposed to hypoxia had better recovery of respiratory rate when treated with Na_2S[9], and it protected from calcium induced mitochondrial swelling[83].

NaHS protected pancreatic beta-cells from H_2O_2 and cytokine induced apoptotic cell death[125]. In neuronal cells and astrocytes, NaHS protected oxidative stress and increased GSH levels[10,124]. It is however acknowledged that H_2S can directly react with H_2O_2, so the question is whether the effects seen are caused by direct reaction between the compounds in the medium[126].

In CSE deficient vascular smooth muscle cells, hypoxia causes reduced viability and increased apoptosis and reactive oxygen species (ROS)[127]. In HEK293 cells, adenovirus mediated

overexpression of CSE reduced antimycin-induced ROS- and mitochondrial superoxide production, which could be further reduced by additional NaHS[8]. NaHS reduced cell death and LDH release after hypoxia, while inhibition of CSE using PAG increased these parameters[88].

Interestingly, the brain seems to be the organ where the protective effects of H_2S are not as unequivocal. This could be due to a difference in H_2S catabolism in the brain, which could influence the results. Also, it might be that the brain has another type of metabolism which is differentially affected by H_2S, causing reduced protection or even increased damage. That the blood-brain barrier plays a role in this seems unlikely, since H_2S can diffuse over lipid membranes without a transporter or channel. However, publication bias could be a factor here as well, with negative studies on H_2S in other organs not being published causing an overly positive view on the effects of H_2S. The vast amount of literature on H_2S in the ischemic setting points toward a highly protective role of exogenous and endogenous H_2S. Modulation of the amount of oxidative stress through sequestration of oxidants and modulation of mitochondrial ROS-production play an important role in the protection against hypoxic insults. In addition, the modulation of metabolism and reduction in oxygen demand of the end organ hold great promise for future applications. In addition, H_2S modulates the secondary effects of ischemia, such as inflammation, apoptosis and fibrosis. These properties make it a very promising substance for the protection of organs against ischemia in the clinical setting.

MECHANISMS OF THE ANTIOXIDATIVE PROPERTIES OF H_2S

The major proposed mechanism behind the protective effects of H_2S in models of oxidative stress is the direct scavenging of reactive oxygen species and the increase in GSH levels that can be achieved, increasing the antioxidative capacity of the cell. NaHS directly scavenges O_2^- and H_2O_2[126], and it protects cells from the cytotoxic lipid oxidation product 4-hydroxyneonal (4-HNE), likely by scavenging[128]. Also, NaHS activates ROS scavenger CuZn-SOD in vitro and in a cell free system[118]. Many studies have shown the positive effects H_2S has on intracellular GSH levels.

Na_2S preserves mitochondrial integrity and oxygen consumption after myocardial ischemia[9], and attenuated mitochondrial dysfunction during the development of ischemia-induced heart failure[86]. The effects of NaHS were abolished in cells lacking mitochondria[58]. Modulation of mitochondrial activity might reduce the amount of mitochondrial superoxide production during stress.

Induction of a hypometabolic state is one of the most fascinating aspects of H_2S[59]. When given in subtoxic concentrations in gaseous form, mice exhibit a reversible hibernation-like state, showing >90% reduction in metabolic rate and core body temperature declines to near ambient temperature. This state has been shown to be highly protective, independent from the associated hypothermia[60,62,85,101]. However, up until now, mixed results have been attained when trying to translate these results in rodents to larger mammals[129-131].

Another mechanism might be H_2S acting as an electron donor to the mitochondrial electron transport chain during periods of low oxygen availability[26,132], causing at least some production of ATP for maintenance of cellular processes during hypoxia. Interestingly, CSE can translocate to the mitochondria upon cellular stress, where it can affect metabolism[28], while MPST can also be localized to mitochondria[26].

The ATP-sensitive K^+ channel (K_{ATP}) allows potassium to move to the intracellular compartment when opened. A great amount of studies show that the effects of H_2S on vascular tone are at least partly dependent on activation of K_{ATP} channels (see the extensive review by Rui Wang[133]). Recent experiments have indicated that H_2S can directly sulfhydrate the K_{ATP} channel and thereby activate it[6]. The reduction in infarct size in a model of rat MI was attenuated by inhibition of mitochondrial K_{ATP} channels[78]. In neurons exposed to hypoxia, the protective effects of NaHS were inhibited by K_{ATP} channel blockade but not by specific mitochondrial K_{ATP} channel blockade[121].

Another possible mechanism is the stimulation of angiogenesis and the formation of collaterals. H_2S increased the amount of collaterals formed 4 weeks after MI[82], and after rat hind limb ischemia, NaHS increased regional blood flow and angiographic score after 4 weeks, with increased collateral formation, capillary density and vascular endothelial growth factor (VEGF) expression[134]. CSE siRNA blocked VEGF induced endothelial cell migration[13]. In addition, wound healing is impaired in $CSE^{-/-}$ mice, and in aortic rings of $CSE^{-/-}$ animals, microvessel formation was reduced compared to WT, and could not be stimulated by VEGF[13]. These effects are probably not responsible for the effects of H_2S on the short term, but might be an additional route of beneficial properties .

The similarities between H_2S and NO / CO as gasotransmitters have led to the hypothesis that the effects of H_2S might be mediated through NO and its synthetizing enzymes eNOS and iNOS. In $eNOS^{-/-}$ mice, Na_2S failed to improve survival after CA/CPR, although $eNOS^{-/-}$ mice are more susceptible to CA/CPR so small effects might have been overlooked with the larger mortality and the relatively small amount of animals used[83]. NaHS induced eNOS and in human microvascular endothelial cells. Inhibition of NOS partially reduced the effects of NaHS on leukocyte rolling and adhesion, while in $eNOS^{-/-}$ animals leukocyte rolling was not affected by NaHS but adhesion was[115]. In all, there seems to be some interaction between H_2S and NO, but which part of the effects of H_2S are mediated or modulated through NO has yet to be determined.

Heme oxygenase 1 (HO-1) is an enzyme involved in the catabolism of heme, and in the process produces CO. It has shown protective and antioxidant effects in many models[135]. Inhibition of HO-1 or deficiency in $HO\text{-}1^{-/-}$ mice abolished the effects of NaHS on leukocyte rolling and adhesion after intestinal IRI, while activation of HO-1 had similar effects to NaHS[136]. In $Nrf2^{-/-}$ animals, Na_2S pretreatment (single injection 24 hours before ischemia) failed to increase HO-1 expression as it did in wildtype animals, and the protective effects of Na_2S in MI were abolished[76]. This shows that the effects of H_2S pretreatment could be mediated through Nrf2 mediated expression of protective proteins. The direct effects of H_2S when given directly before or after ischemia might be regulated differently. Also, another study indicated that HO-1 expression was not associated with H_2S-mediated protection[101].

Heat shock protein 90 (HSP90) has been implicated as a protective protein that can stabilize intracellular proteins and is involved in apoptosis[137]. NaHS protected against $CoCl_2$-induced ROS, cell death and apoptosis, but not when combined with HSP90 inhibition in rat pheochromocytoma cells[138]. In a model of neuronal hypoxia, NaHS increased HSP90 expression, while inhibition of HSP90 abolishes the protective effects of NaHS[121]. These data indicate that the effects of H_2S might be partly mediated by HSP90 activation.

Table 2 - Proposed mechanisms for protective effects of H_2S.

Direct scavenging of reactive oxygen/nitrogen species
Increase in GSH levels
Reduction of mitochondrial superoxide production
Activation of ROS-scavengers (e.g. MnSOD, CuZnSOD)
Preservation of mitochondrial integrity
Acting as an electron donor in the electron transport chain
Modulation of mitochondrial activity / Hypometabolism
Induction of hypothermia
Modulation of vascular proliferation
Modulation of leukocyte adhesion / rolling
Modulation of cytokine production
Activation of K_{ATP}-channels
Activation of calcium activated K^+ channels
Modulation of protein activity through sulfhydration
Modulation of platelet function
Modulation of hypoxia inducible factor (HIF)
Modulation of Nrf2 signaling
Increase in eNOS / iNOS / HO-1
Increase in HSP90
Modulation of COX-2 activity
Modulation of GSK3beta activity
Modulation of apoptotic signaling
Modulation of autophagy

H_2S MEASUREMENT

The measurement of endogenous levels of H_2S poses a large problem for the research community, since the volatility, reactivity and gaseous form make it difficult to assess the levels of H_2S that are present intracellularly, extracellularly or in serum. The exact concentrations that are present in human serum have been a topic of heated discussion in the past years. For example, some argue that the most widely published levels (between 10 and 100 µM) would cause human blood to heavily smell like H_2S[139,140]. Experiments that have used very high concentrations thusly need to be interpreted with caution. However, the volatile nature of H_2S at physiological pH, the ability to react with proteins and organic substances intracellularly, the capability to diffuse over membranes without transporter molecules[141] and the general difficulty of measurement of the substance make it difficult to interpret any result on the measurement of H_2S.

A recent advance that could change this is the development of fluorescent probes that are sensitive to H_2S and that can be loaded into living cells. This field seems to have exploded in the past two years, catering to the need for measurement of intracellular H_2S levels and changes,

where it likely matters most. Table 3 gives an overview of the probes that have been published in the past years, showing 24 separate probes that have been reported since 2011. No similar probes have been reported before 2011 according to our research.

These probes are very interesting for providing insights in the changes of intracellular H_2S. Most of these probes can be used in live cells for fluorescence imaging. One of the obstacles now is the slow reactivity of the probes to H_2S, making them unusable for measuring fast changes in H_2S levels. However, at least one of the published probes has a reaction time of around 30 seconds, indicating that there is progress on this aspect[142].

TRANSLATION TO THERAPEUTIC APPLICATIONS

Therapeutic window of H_2S dose

Some studies have shown that the effect of H_2S can be very dose dependent, with too high doses showing no or even detrimental effects. In porcine cardiac arrest and resuscitation, high dose Na_2S (1mg/kg) worsened cerebral damage and levels of troponin T, while 0.3 mg/kg had no effect[143]. In a model of partial hepatic ischemia, Na_2S treatment (0.3 and 1 mg/kg) protected from liver damage, while 2 mg/kg increased damage[102]. These results infer that before clinical translation, meticulous dose-finding studies are required and possibly reliable serum-level measurement needs to be developed to monitor treatment.

Timing of treatment

Many different treatment protocols have been used, and one of the most variable parameters was the timing of the treatment with H_2S. For example, NaHS injection 1 day prior to MI in rats decreased infarct size, left ventricle internal diameter and anterior wall thickness. Injection of NaHS 3 days prior to MI also showed protective effects, while NaHS 5 days prior to MI had no effect. Posttreatment (after MI, at day 0,1 and 2) had effects, but less when compared to pretreatment, and combination treatment had no additional effect[144]. In another study, single administration of Na_2S did not affect IRI induced cardiac function loss, while daily administration for 1 week did[86]. Last, NaHS at 24 hours pre-ischemia, but not at 1 hour before ischemia or during reperfusion, reduced leukocyte rolling and adhesion post reperfusion[76]. This might be related to the activation of transcription factors such as Nrf2, since the preconditioning effect of H_2S in cardiac MI is absent in $Nrf2^{-/-}$ mice[76]. It is likely that the effects of H_2S when given more than a couple of hours before ischemia are mediated through increasing the expression of other protective proteins, such as HO-1 in a Nrf2-dependent pathway[76], while the more direct effects of H_2S are mediated through some other mechanism since H_2S levels are only elevated a short amount of time after bolus injection. Interestingly, CSE upregulation can also be dependent on Nrf2 signaling[35], as can HSP90 and HO-1[76].

Pretreatment and posttreatment have both been effective in ischemic models, in some models pretreatment showed to be more effective[62]. However, pretreatment is not clinically applicable in many situations, such as acute myocardial infarction or shock. In those models only posttreatment with H_2S can be used since the patient only presents after the onset of ischemia. In the clinical setting this would mean oral or intravenous application, or injection into the organ itself (e.g. intramyocardial injection).

Table 3 - H_2S Probes - Overview table.

Probe	Livecells	Properties	Spectrum	Reference
NIR-H_2S	Yes	Cytoplasmatic fluorescence. Reacts with L-cysteine and GSH in cell-free system.	Ex: 650 nm / Em: 708 nm	Cao (2012)
C-7Az	Yes	Cytoplasmatic fluorescence. Can be used for formalin fixed tissue sections.	Ex: 340 nm / Em: 450 nm	B Chen (2012)
cpGFP-Tyr66pAzF	Yes	Genetically encoded proteins, expressed through transfection. Does not react with L-cysteine or GSH.	Ex: 480 nm / Em: 510 nm	S Chen (2012)
CouMC	Yes	Mitochondrial fluorescence. Short response time (<30s).	Ex: 475 nm / Em: 510 / 652 nm	Y Chen (2013)
FS1	Yes	Can be used in live tissue using two-photon imaging, cytoplasmatic staining.	Ex: 363 nm / Em: 548 nm	Das (2012)
$Tp^{Ph,Me}$ Zn(Mus) 2	Unknown	On H_2S exposure, probe decreases in fluorescence and changes absorbance on exposure to H_2S	Ex: 360 nm / Em: 434 nm Absorbance at 354 and 383 nm	Galardon (2012)
L1Cu	Yes	Can be used in live cells, dependent on intracellular Cu(II).	Ex: 494 nm / Em: 523 nm	Hou (2012)
Li et al. probe 1b	Yes	Cytoplasmatic fluorescence. Reacts with L-cysteine and GSH in cell-free system.	Ex: 380 nm / Em: 515 nm	Li (2012)
SF1	Yes	Cytoplasmatic fluorescence. Reacts with NO and O_2^- in cell-free system.	Ex: 492 nm / Em: 525 nm	Lippert (2011)
SF2	Yes	Cytoplasmatic fluorescence. Reacts with Na_2SO_3 and O_2^- in cell-free system.	Ex: 490 nm / Em: 525 nm	Lippert (2011)
Liu et al. probe 6	Yes	Cytoplasmatic and nuclear fluorescence. Does not react with L-cysteine or GSH.	Ex: 476 nm / Em: 513 nm	Liu (2012)
HSN2	Yes	Cytoplasmatic fluorescence. Does not react with L-cysteine or GSH.	Ex: 432 nm / Em: 542 nm	Montoya (2012)
DNS-Az	Unknown	Does not react to other anions, used for measurement of H_2S concentration in blood.	Ex: 340 nm / Em: 535 nm	Peng (2011)

Table 3 - Continued

Probe	Live cells	Properties	Spectrum	Reference
SFP-1	Yes	Cytoplasmatic fluorescence. Detects H_2S produced from CBS in cell free reaction. Reacts with GSH and L-cysteine.	Ex: 300 nm / Em: 388 nm	Qian (2012)
SFP-2	Yes	Cytoplasmatic fluorescence. Reacts slightly with GSH and 2-mercaptoethanol in cell free system.	Ex: 465 nm / Em: 510 nm	Qian (2012)
HSip-1	Yes	Cytoplasmatic and nuclear fluorescence. Detects H_2S production from MPST. No reaction to ROS, GSH or L-cysteine.	Ex: 491 nm / Em: 516 nm	Sasakura (2011)
551	Unknown	Sensitive micelle system. Reacts with $S_2O_4^{2-}$, $S_2O_5^{2-}$, HSO_3^-	Ex: 404 nm / Em: 540 nm	Tian (2013)
Wan et al. probe 1	Yes	Cytoplasmatic fluorescence. Can be used in living zebrafish.	Ex: 535 nm / Em: 620 nm	Wan (2013)
Cy-NO_2	Yes	Cytoplasmatic fluorescence. Hyghly specific in cell free system.	Ex: 755 nm / Em: 789 nm	Wang (2012)
Xu et al. probe E1	Yes	Staining pattern seems nuclear. Does not react with GSH, L-cysteine or homocysteine in cell free system.	Ex: 295 nm / Em: 487 nm	Xu (2012)
Xuan et al. probe 1	Yes	Cytoplasmatic fluorescence. Reacts with NADH but not ROS, L-cysteine or GSH. Slow kinetics.	Ex: 440 nm / Em: 544 nm	Xuan (2012)
Yu et al. probe	Yes	Carbon dot based. Cytoplasmatic fluorescence.	Ex: 425 nm / Em: 526 nm	Yu (2012)
Zhao et al. probe1	Unknown	Absorbance reduces on reaction with H_2S. Reacts to GSH and L-cysteine in cell free system.	Absorbance at 527 nm	Zhao (2012)
PI-N_3	Yes	Cytoplasmatic fluorescence. Reacts with L-cysteine and GSH in cell-free system.	Ex: 358 nm / Em: 423 nm	Zheng (2012)

Other situations comprise predictable moments of ischemia, mainly during surgical interventions such as organ transplantation, momentary aortic occlusion or temporary occlusion of cerebral arteries during the clipping of cerebral aneurysms. In the setting of organ transplantation, the brain dead organ donor can be treated with intravenous or gaseous H_2S. Also, the organ can be exposed to H_2S during machine perfusion, a treatment system which has already shown clinical potential in protecting donor organs[145,146]. Last, the organ recipient can be treated with H_2S post-transplantation.

Mode of administration

Experimental studies have shown many methods of administration for H_2S treatment, and each has its advantages and disadvantages for eventual clinical applications. Gaseous H_2S is difficult to handle safely, corrosive, toxic in higher concentrations and very malodorous. This means it can only be applied in well ventilated rooms or in intubated persons with specialized equipment. It can however cause very long and stable hypometabolic states in mice[59,60], indicating that long term stable levels are reached with gaseous H_2S.

The soluble sulfide sodium salts (NaHS and Na_2S) have very rapid dynamics, which makes it hard to achieve steady concentrations in vivo[53,56]. Continuous intravenous administration might achieve constant levels, but has shown a small therapeutic window in experimental studies. Slow releasing donors of H_2S are in development and could counter some of these problems, but remain to be proven safe for human use as of this moment. Offering H_2S through the drinking water, as has been done in experimental settings, is not recommended in the human situation because of the horrendous taste and smell. One new development that has shown potential is the use of naturally occurring cysteine analogues, such as DATS and DADS. These can increase H_2S levels over longer periods of time, and could be taken orally. Also, DATS and DADS can be derived from garlic, so large amounts of garlic or maybe even garlic concentrates could be used as a food supplement.

Last is the modulation of endogenous production, which is at the moment problematic, since the best known modulators are inhibitors of CSE and CBS, while most studies show that the activity of these proteins needs to be increased to be beneficial. Adenovirus mediated overexpression is an option, but no groups are investigating this for human applications to the best of our knowledge. Modulation of Ethe1 or CDO is only hypothetical at the moment, and the detrimental effects of the loss of Ethe1 do not bode well for long term modulation of this protein[43].

In all, H_2S is a highly promising molecule that has only given us a small peek into its broad functionality. The protective effects in ischemia and oxidative stress are strong, and the development of H_2S based therapeutics could greatly improve outcome of surgically induced ischemia and ischemic disease.

REFERENCES

1. Mani, S., Yang, G. & Wang, R. A critical life-supporting role for cystathionine γ-lyase in the absence of dietary cysteine supply. *Free Radic Biol Med* 50, 1280–1287 (2011).
2. Ishii, I. *et al.* Cystathionine gamma-Lyase-deficient mice require dietary cysteine to protect against acute lethal myopathy and oxidative injury. *Journal of Biological Chemistry* 285, 26358–26368 (2010).
3. Yang, G. *et al.* H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322, 587–590 (2008).
4. Huang, P. L. *et al.* Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377, 239–242 (1995).
5. Zhao, W., Zhang, J., Lu, Y. & Wang, R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. *EMBOJ.* 20, 6008–6016 (2001).
6. Mustafa, A. K. *et al.* Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. *Circ. Res.* 109, 1259–1268 (2011).
7. Morikawa, T. *et al.* Hypoxic regulation of the cerebral microcirculation is mediated by a carbon monoxide-sensitive hydrogen sulfide pathway. *Proc Natl Acad Sci USA* 109, 1293–1298 (2012).
8. Bos, E. M. *et al.* Cystathionine γ-Lyase Protects against Renal Ischemia/Reperfusion by Modulating Oxidative Stress. *J Am Soc Nephrol* 24, 759–770 (2013).
9. Elrod, J. W. *et al.* Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proc Natl Acad Sci USA* 104, 15560–15565 (2007).
10. Kimura, Y., Goto, Y.-I. & Kimura, H. Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxid Redox Signal* 12, 1–13 (2010).
11. Robert, K. *et al.* Cystathionine beta synthase deficiency promotes oxidative stress, fibrosis, and steatosis in mice liver. *Gastroenterology* 128, 1405–1415 (2005).
12. Bearden, S. E., Beard, R. S. & Pfau, J. C. Extracellular transsulfuration generates hydrogen sulfide from homocysteine and protects endothelium from redox stress. *Am J Physiol Heart Circ Physiol* 299, H1568–76 (2010).
13. Papapetropoulos, A. *et al.* Hydrogen sulfide is an endogenous stimulator of angiogenesis. *Proc Natl Acad Sci USA* 106, 21972–21977 (2009).
14. Coletta, C. *et al.* Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. *Proc Natl Acad Sci USA* 109, 9161–9166 (2012).
15. Suzuki, K. *et al.* Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function. *Proc Natl Acad Sci USA* 108, 13829–13834 (2011).
16. Yang, G., Tang, G., Zhang, L., Wu, L. & Wang, R. The pathogenic role of cystathionine γ-lyase/hydrogen sulfide in streptozotocin-induced diabetes in mice. *Am J Pathol* 179, 869–879 (2011).
17. Zhang, L., Yang, G., Tang, G., Wu, L. & Wang, R. Rat pancreatic level of cystathionine γ-lyase is regulated by glucose level via specificity protein 1 (SP1) phosphorylation. *Diabetologia* 54, 2615–2625 (2011).
18. Kundu, S. *et al.* Nitrotyrosinylation, remodeling and endothelial-myocyte uncoupling in iNOS, cystathionine beta synthase (CBS) knockouts and iNOS/CBS double knockout mice. *J. Cell. Biochem.* 106, 119–126 (2009).
19. Kumar, M. *et al.* GABAA receptor agonist mitigates homocysteine-induced cerebrovascular remodeling in knockout mice. *Brain Research* 1221, 147–153 (2008).
20. Watanabe, M. *et al.* Mice deficient in cystathionine beta-synthase: animal models for mild and severe homocyst(e)inemia. *Proc Natl Acad Sci USA* 92, 1585–1589 (1995).
21. Kabil, H., Kabil, O., Banerjee, R., Harshman, L. G. & Pletcher, S. D. Increased transsulfuration mediates longevity and dietary restriction in Drosophila. *Proc Natl Acad Sci USA* 108, 16831–16836 (2011).
22. Ichinohe, A. *et al.* Cystathionine beta-synthase is enriched in the brains of Down's patients. *Biochem Biophys Res Commun* 338, 1547–1550 (2005).
23. Shibuya, N. *et al.* 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxid Redox Signal* 11, 703–714 (2009).
24. Shibuya, N., Mikami, Y., Kimura, Y., Nagahara, N. & Kimura, H. Vascular endothelium expresses 3-mercaptopyruvate sulfurtransferase and

produces hydrogen sulfide. *J Biochem* 146, 623–626 (2009).

25. Markand, S. *et al*. Cystathionine Beta Synthase Expression in Mouse Retina. *Curr. Eye Res.* (2013). doi:10.3109/02713683.2013.774024
26. Módis, K., Coletta, C., Erdelyi, K., Papapetropoulos, A. & Szabó, C. Intramitochondrial hydrogen sulfide production by 3-mercaptopyruvate sulfurtransferase maintains mitochondrial electron flow and supports cellular bioenergetics. *FASEB J* (2012). doi:10.1096/fj.12-216507
27. Goubern, M., Andriamihaja, M., Nübel, T., Blachier, F. & Bouillaud, F. Sulfide, the first inorganic substrate for human cells. *FASEB J* 21, 1699–1706 (2007).
28. Fu, M. *et al*. Hydrogen sulfide (H2S) metabolism in mitochondria and its regulatory role in energy production. *Proc Natl Acad Sci USA* 109, 2943–2948 (2012).
29. Yin, P. *et al*. Sp1 is involved in regulation of cystathionine γ-lyase gene expression and biological function by PI3K/Akt pathway in human hepatocellular carcinoma cell lines. *Cell. Signal.* 24, 1229–1240 (2012).
30. Wu, N., Siow, Y. L. & O, K. Ischemia/reperfusion reduces transcription factor Sp1-mediated cystathionine beta-synthase expression in the kidney. *J Biol Chem* 285, 18225–18233 (2010).
31. Yang, G., Pei, Y., Teng, H., Cao, Q. & Wang, R. Specificity protein-1 as a critical regulator of human cystathionine gamma-lyase in smooth muscle cells. *Journal of Biological Chemistry* 286, 26450–26460 (2011).
32. Yang, G., Pei, Y., Cao, Q. & Wang, R. MicroRNA-21 represses human cystathionine gamma-lyase expression by targeting at specificity protein-1 in smooth muscle cells. *Journal of cellular physiology* 227, 3192–3200 (2012).
33. Sen, N. *et al*. Hydrogen sulfide-linked sulfhydration of NF-κB mediates its antiapoptotic actions. *Mol. Cell* 45, 13–24 (2012).
34. Kaspar, J. W., Niture, S. K. & Jaiswal, A. K. Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radic Biol Med* 47, 1304–1309 (2009).
35. Hassan, M. I. *et al*. Platelet-derived growth factor-BB induces cystathionine γ-lyase expression in rat mesangial cells via a redox-dependent mechanism. *Br. J. Pharmacol.* 166, 2231–2242 (2012).
36. Renga, B., Mencarelli, A., Migliorati, M., Distrutti, E. & Fiorucci, S. Bile-acid-activated farnesoid X receptor regulates hydrogen sulfide production and hepatic microcirculation. *World J Gastroenterol* 15, 2097–2108 (2009).
37. Agrawal, N. & Banerjee, R. Human Polycomb 2 Protein Is a SUMO E3 Ligase and Alleviates Substrate-Induced Inhibition of Cystathionine beta-Synthase Sumoylation. *PLoS ONE* 3, e4032 (2008).
38. Shibuya, N. *et al*. A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. *Nat Commun* 4, 1366 (2013).
39. Mikami, Y. *et al*. Hydrogen sulfide protects the retina from light-induced degeneration by the modulation of Ca2+ influx. *Journal of Biological Chemistry* 286, 39379–39386 (2011).
40. Mikami, Y. *et al*. Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. *Biochem J* 439, 479–485 (2011).
41. Roman, H. B. *et al*. The Cysteine Dioxygenase Knockout Mouse: Altered Cysteine Metabolism in Nonhepatic Tissues Leads to Excess H2S/HS− Production and Evidence of Pancreatic and Lung Toxicity. *Antioxid Redox Signal* (2013). doi:10.1089/ars.2012.5010
42. Ueki, I. *et al*. Knockout of the murine cysteine dioxygenase gene results in severe impairment in ability to synthesize taurine and an increased catabolism of cysteine to hydrogen sulfide. *Am J Physiol Endocrinol Metab* 301, E668–84 (2011).
43. Tiranti, V. *et al*. Loss of ETHE1, a mitochondrial dioxygenase, causes fatal sulfide toxicity in ethylmalonic encephalopathy. *Nat Med* 15, 200–205 (2009).
44. Di Meo, I. *et al*. Chronic exposure to sulfide causes accelerated degradation of cytochrome c oxidase in ethylmalonic encephalopathy. *Antioxid Redox Signal* 15, 353–362 (2011).
45. Viscomi, C. *et al*. Combined treatment with oral metronidazole and N-acetylcysteine is effective in ethylmalonic encephalopathy. *Nat Med* 16, 869–871 (2010).
46. Paul, B. D. & Snyder, S. H. H_2S signalling through protein sulfhydration and beyond. *Nat Rev Mol Cell Biol* 13, 499–507 (2012).
47. Mustafa, A. K. *et al*. H2S signals through protein S-sulfhydration. *Sci Signal* 2, ra72 (2009).
48. Krishnan, N., Fu, C., Pappin, D. J. & Tonks, N. K. H2S-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. *Sci Signal* 4, ra86 (2011).

49. Hayes, J. D., Hourihan, J. M. & Kenna, J. G. The gasotransmitter hydrogen sulfide induces Nrf2-target genes by inactivating the Keap1 ubiquitin ligase substrate adaptor through formation of a disulfide bond between Cys-226 and Cys-613. *Antioxid Redox Signal* (2012). doi:10.1089/ars.2012.4944
50. Tao, B.-B. *et al*. VEGFR2 Functions As an H(2) S-Targeting Receptor Protein Kinase with Its Novel Cys1045-Cys1024 Disulfide Bond Serving As a Specific Molecular Switch for Hydrogen Sulfide Actions in Vascular Endothelial Cells. *Antioxid Redox Signal* (2013). doi:10.1089/ars.2012.4565
51. Nishida, M. *et al*. Hydrogen sulfide anion regulates redox signaling via electrophile sulfhydration. *Nat. Chem. Biol*. 8, 714–724 (2012).
52. Li, L. *et al*. Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. *Free Radic Biol Med* 42, 706–719 (2007).
53. Marutani, E. *et al*. A Novel Hydrogen Sulfide-releasing N-Methyl-D-Aspartate Receptor Antagonist Prevents Ischemic Neuronal Death. *Journal of Biological Chemistry* 287, 32124–32135 (2012).
54. Predmore, B. L. *et al*. The polysulfide diallyl trisulfide protects the ischemic myocardium by preservation of endogenous hydrogen sulfide and increasing nitric oxide bioavailability. *Am J Physiol Heart Circ Physiol* 302, H2410–8 (2012).
55. Kimura, Y., Dargusch, R., Schubert, D. & Kimura, H. Hydrogen sulfide protects HT22 neuronal cells from oxidative stress. *Antioxid Redox Signal* 8, 661–670 (2006).
56. Li, L. *et al*. Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide. *Circulation* 117, 2351–2360 (2008).
57. Zhao, Y., Wang, H. & Xian, M. Cysteine-Activated Hydrogen Sulfide (H2S) Donors. *J. Am. Chem. Soc*. 133, 15–17 (2011).
58. Kai, S. *et al*. Hydrogen sulfide inhibits hypoxia- but not anoxia-induced hypoxia-inducible factor 1 activation in a von hippel-lindau- and mitochondria-dependent manner. *Antioxid Redox Signal* 16, 203–216 (2012).
59. Blackstone, E., Morrison, M. & Roth, M. B. H2S induces a suspended animation-like state in mice. *Science* 308, 518 (2005).
60. Blackstone, E. & Roth, M. B. Suspended Animation-Like State Protects Mice from Lethal Hypoxia. *Shock* 27, 370–372 (2007).
61. Miller, D. L. & Roth, M. B. Hydrogen sulfide increases thermotolerance and lifespan in Caenorhabditis elegans. *Proc Natl Acad Sci USA* 104, 20618–20622 (2007).
62. Bos, E. M. *et al*. Hydrogen sulfide-induced hypometabolism prevents renal ischemia/reperfusion injury. *J Am Soc Nephrol* 20, 1901–1905 (2009).
63. Pircher, J. *et al*. Hydrogen Sulfide-Releasing Aspirin Derivative ACS14 Exerts Strong Antithrombotic Effects In Vitro and In Vivo. *Arterioscler Thromb Vasc Biol* (2012). doi:10.1161/ATVBAHA.112.300627
64. Wallace, J. L., Caliendo, G., Santagada, V. & Cirino, G. Markedly reduced toxicity of a hydrogen sulphide-releasing derivative of naproxen (ATB-346). *Br. J. Pharmacol*. 159, 1236–1246 (2010).
65. Blackler, R., Syer, S., Bolla, M., Ongini, E. & Wallace, J. L. Gastrointestinal-sparing effects of novel NSAIDs in rats with compromised mucosal defence. *PLoS ONE* 7, e35196 (2012).
66. Baskar, R., Sparatore, A., del Soldato, P. & Moore, P. K. Effect of S-diclofenac, a novel hydrogen sulfide releasing derivative inhibit rat vascular smooth muscle cell proliferation. *Eur. J. Pharmacol*. 594, 1–8 (2008).
67. Rossoni, G. *et al*. The hydrogen sulphide-releasing derivative of diclofenac protects against ischaemia-reperfusion injury in the isolated rabbit heart. *Br. J. Pharmacol*. 153, 100–109 (2008).
68. Isenberg, J. S. *et al*. Modulation of angiogenesis by dithiolethione-modified NSAIDs and valproicacid. *Br. J. Pharmacol*. 151, 63–72 (2007).
69. Shukla, N. *et al*. Effect of hydrogen sulphide-donating sildenafil (ACS6) on erectile function and oxidative stress in rabbit isolated corpus cavernosum and in hypertensive rats. *BJU Int* 103, 1522–1529 (2009).
70. Muzaffar, S. *et al*. H2S-donating sildenafil (ACS6) inhibits superoxide formation and gp91phox expression in arterial endothelial cells: role of protein kinases A and G. *Br. J. Pharmacol*. 155, 984–994 (2008).
71. Lee, M. *et al*. Effects of hydrogen sulfide-releasing L-DOPA derivatives on glial activation: potential for treating Parkinson disease. *J Biol Chem* 285, 17318–17328 (2010).
72. Benavides, G. A. *et al*. Hydrogen sulfide mediates the vasoactivity of garlic. *Proc Natl Acad Sci USA* 104, 17977–17982 (2007).
73. Wang, Q., Wang, X.-L., Liu, H.-R., Rose, P. & Zhu, Y.-Z. Protective effects of cysteine

analogues on acute myocardial ischemia: novel modulators of endogenous H(2)S production. *Antioxid Redox Signal* 12, 1155–1165 (2010).

74. Wang, Q., Liu, H.-R., Mu, Q., Rose, P. & Zhu, Y.-Z. S-propargyl-cysteine protects both adult rat hearts and neonatal cardiomyocytes from ischemia/hypoxia injury: the contribution of the hydrogen sulfide-mediated pathway. *J. Cardiovasc. Pharmacol.* 54, 139–146 (2009).
75. Jensen, K. K. *et al*. Pharmacological activation and genetic manipulation of cystathionine beta-synthase alter circulating levels of homocysteine and hydrogen sulfide in mice. *Eur. J. Pharmacol.* 650, 86–93 (2011).
76. Calvert, J. W. *et al*. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. *Circ. Res.* 105, 365–374 (2009).
77. Pan, T.-T., Feng, Z.-N., Lee, S. W., Moore, P. K. & Bian, J.-S. Endogenous hydrogen sulfide contributes to the cardioprotection by metabolic inhibition preconditioning in the rat ventricular myocytes. *J. Mol. Cell. Cardiol.* 40, 119–130 (2005).
78. Sivarajah, A. *et al*. Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. *Shock* 31, 267–274 (2009).
79. Sodha, N. R. *et al*. Hydrogen sulfide therapy attenuates the inflammatory response in a porcine model of myocardial ischemia/reperfusion injury. *The Journal of Thoracic and Cardiovascular Surgery* 138, 977–984 (2009).
80. Sodha, N. R. *et al*. The effects of therapeutic sulfide on myocardial apoptosis in response to ischemia-reperfusion injury. *Eur J Cardiothorac Surg* 33, 906–913 (2008).
81. Yao, L.-L. *et al*. Hydrogen sulfide protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by preventing GSK-3beta-dependent opening of mPTP. *Am J Physiol Heart Circ Physiol* 298, H1310–9 (2010).
82. Qipshidze, N., Metreveli, N., Mishra, P. K., Lominadze, D. & Tyagi, S. C. Hydrogen Sulfide Mitigates Cardiac Remodeling During Myocardial Infarction via Improvement of Angiogenesis. *Int. J. Biol. Sci.* 8, 430–441 (2012).
83. Minamishima, S. *et al*. Hydrogen sulfide improves survival after cardiac arrest and cardiopulmonary resuscitation via a nitric oxide synthase 3-dependent mechanism in mice. *Circulation* 120, 888–896 (2009).
84. Szabó, G. *et al*. Cardioprotective effects of hydrogen sulfide. *Nitric Oxide* 25, 201–210 (2011).
85. Snijder, P. M. *et al*. Gaseous Hydrogen Sulfide Protects against Myocardial Ischemia-Reperfusion Injury in Mice Partially Independent from Hypometabolism. *PLoS ONE* 8, e63291 (2013).
86. Calvert, J. W. *et al*. Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. *Circulation* 122, 11–19 (2010).
87. Zhuo, Y. *et al*. Cardioprotective effect of hydrogen sulfide in ischemic reperfusion experimental rats and its influence on expression of survivin gene. *Biol Pharm Bull* 32, 1406–1410 (2009).
88. Zhu, Y. Z. *et al*. Hydrogen sulfide and its possible roles in myocardial ischemia in experimental rats. *J Appl Physiol* 102, 261–268 (2006).
89. Zhu, J. X. G. *et al*. Detrimental effects of prolonged warm renal ischaemia-reperfusion injury are abrogated by supplemental hydrogen sulphide: an analysis using real-time intravital microscopy and polymerase chain reaction. *BJU Int* (2012). doi:10.1111/j.1464-410X.2012.11555.x
90. Xu, Z. *et al*. Ischemia-reperfusion reduces cystathionine-beta-synthase-mediated hydrogen sulfide generation in the kidney. *Am J Physiol Renal Physiol* 297, F27–35 (2009).
91. Simon, F. *et al*. Effects of intravenous sulfide during porcine aortic occlusion-induced kidney ischemia/reperfusion injury. *Shock* 35, 156–163 (2011).
92. Tripatara, P. *et al*. Characterisation of cystathionine gamma-lyase/hydrogen sulphide pathway in ischaemia/reperfusion injury of the mouse kidney: An in vivo study. *Eur. J. Pharmacol.* 606, 205–209 (2009).
93. Tripatara, P. *et al*. Generation of endogenous hydrogen sulfide by cystathionine gamma-lyase limits renal ischemia/reperfusion injury and dysfunction. *Lab. Invest.* 88, 1038–1048 (2008).
94. Florian, B. *et al*. Long-term hypothermia reduces infarct volume in aged rats after focal ischemia. *Neuroscience Letters* 438, 180–185 (2008).
95. Li, Z., Wang, Y., Xie, Y., Yang, Z. & Zhang, T. Protective effects of exogenous hydrogen sulfide on neurons of hippocampus in a rat model of brain ischemia. *Neurochem Res* 36, 1840–1849 (2011).
96. Ren, C. *et al*. Dynamic change of hydrogen sulfide during global cerebral ischemia-reperfusion and its effect in rats. *Brain Research* 1345, 197–205 (2010).

97. Qu, K., Chen, C. P. L. H., Halliwell, B., Moore, P. K. & Wong, P. T. H. Hydrogen sulfide is a mediator of cerebral ischemic damage. *Stroke* 37, 889–893 (2006).

98. Knapp, J. *et al.* Hypothermia and neuroprotection by sulfide after cardiac arrest and cardiopulmonary resuscitation. *Resuscitation* 82, 1076–1080 (2011).

99. Grobelny, B. T. *et al.* Gain-of-function polymorphisms of cystathionine β-synthase and delayed cerebral ischemia following aneurysmal subarachnoid hemorrhage. *J Neurosurg* 115, 101–107 (2011).

100. Chen, Y., Liu, Z. & Xie, X. Hydrogen sulphide attenuates renal and cardiac injury after total hepatic ischemia and reperfusion. *J Surg Res* 164, e305–13 (2010).

101. Bos, E. M. *et al.* Beneficial effects of gaseous hydrogen sulfide in hepatic ischemia/reperfusion injury. *Transpl Int* 25, 897–908 (2012).

102. Jha, S., Calvert, J. W., Duranski, M. R., Ramachandran, A. & Lefer, D. J. Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. *Am J Physiol Heart Circ Physiol* 295, H801–6 (2008).

103. Kang, K. *et al.* Role of hydrogen sulfide in hepatic ischemia-reperfusion-induced injury in rats. *Liver Transpl* 15, 1306–1314 (2009).

104. Wang, D. *et al.* The role of AKT1 and autophagy in the protective effect of hydrogen sulphide against hepatic ischemia/reperfusion injury in mice. *Autophagy* 8, 954–962 (2012).

105. Morrison, M. L. *et al.* Surviving blood loss using hydrogen sulfide. *J Trauma* 65, 183–188 (2008).

106. Ganster, F. *et al.* Effects of hydrogen sulfide on hemodynamics, inflammatory response and oxidative stress during resuscitated hemorrhagic shock in rats. *Critical care (London, England)* 14, R165 (2010).

107. Bracht, H. *et al.* Effects of intravenous sulfide during resuscitated porcine hemorrhagic shock*. *Crit. Care Med.* 40, 2157–2167 (2012).

108. Drabek, T. Hydrogen sulfide-curiouser and curiouser!*. *Crit. Care Med.* 40, 2255–2256 (2012).

109. George, T. J. *et al.* Hydrogen sulfide decreases reactive oxygen in a model of lung transplantation. *J Surg Res* 178, 494–501 (2012).

110. George, T. J. *et al.* Inhaled hydrogen sulfide improves graft function in an experimental model of lung transplantation. *J Surg Res* 178, 593–600 (2012).

111. Fu, Z., Liu, X., Geng, B., Fang, L.-P. & Tang, C.-S. Hydrogen sulfide protects rat lung from ischemia-reperfusion injury. *Life Sci.* 82, 1196–1202 (2008).

112. Liu, H., Bai, X.-B., Shi, S. & Cao, Y.-X. Hydrogen sulfide protects from intestinal ischaemia-reperfusion injury in rats. *J Pharm Pharmacol* 61, 207–212 (2009).

113. Yonezawa, D. *et al.* A protective role of hydrogen sulfide against oxidative stress in rat gastric mucosal epithelium. *Toxicology* 241, 11–18 (2007).

114. Henderson, P. W. *et al.* Hydrogen sulfide attenuates intestinal ischemia-reperfusion injury when delivered in the post-ischemic period. *J Gastroenterol Hepatol* 25, 1642–1647 (2010).

115. Yusof, M., Kamada, K., Kalogeris, T., Gaskin, F. S. & Korthuis, R. J. Hydrogen sulfide triggers late-phase preconditioning in postischemic small intestine by an NO- and p38 MAPK-dependent mechanism. *Am J Physiol Heart Circ Physiol* 296, H868–76 (2009).

116. Henderson, P. W. *et al.* Hydrogen sulfide protects against ischemia-reperfusion injury in an in vitro model of cutaneous tissue transplantation. *J Surg Res* 159, 451–455 (2010).

117. Luo, Y. *et al.* Hydrogen sulfide prevents hypoxia-induced apoptosis via inhibition of an H2O2-activated calcium signaling pathway in mouse hippocampal neurons. *Biochem Biophys Res Commun* 425, 473–477 (2012).

118. Sun, W.-H., Liu, F., Chen, Y. & Zhu, Y. C. Hydrogen sulfide decreases the levels of ROS by inhibiting mitochondrial complex IV and increasing SOD activities in cardiomyocytes under ischemia/reperfusion. *Biochem Biophys Res Commun* 421, 164–169 (2012).

119. Chen, S.-L. *et al.* Hydrogen sulphide protects H9c2 cells against chemical hypoxia-induced injury. *Clin Exp Pharmacol Physiol* 37, 316–321 (2010).

120. Yang, C. *et al.* Hydrogen sulfide protects against chemical hypoxia-induced cytotoxicity and inflammation in HaCaT cells through inhibition of ROS/NF-κB/COX-2 pathway. *PLoS ONE* 6, e21971 (2011).

121. Tay, A. S., Hu, L. F., Lu, M., Wong, P. T. H. & Bian, J.-S. Hydrogen sulfide protects neurons against hypoxic injury via stimulation of ATP-sensitive potassium channel/protein kinase C/extracellular signal-regulated kinase/heat shock protein 90 pathway. *Neuroscience* 167, 277–286 (2010).

122. Whiteman, M. *et al*. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? *J Neurochem* 90, 765–768 (2004).
123. Whiteman, M. *et al*. Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? *Biochem Biophys Res Commun* 326, 794–798 (2004).
124. Lu, M., Hu, L.-F., Hu, G. & Bian, J.-S. Hydrogen sulfide protects astrocytes against H(2)O(2)-induced neural injury via enhancing glutamate uptake. *Free Radic Biol Med* 45, 1705–1713 (2008).
125. Taniguchi, S., Kang, L., Kimura, T. & Niki, I. Hydrogen sulphide protects mouse pancreatic β-cells from cell death induced by oxidative stress, but not by endoplasmic reticulum stress. *Br. J. Pharmacol*. 162, 1171–1178 (2011).
126. Geng, B. *et al*. Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol. *Biochem Biophys Res Commun* 318, 756–763 (2004).
127. Bryan, S., Yang, G., Wang, R. & Khaper, N. Cystathionine gamma-lyase-deficient smooth muscle cells exhibit redox imbalance and apoptosis under hypoxic stress conditions. *Exp Clin Cardiol* 16, e36–41 (2011).
128. Schreier, S. M. *et al*. Hydrogen sulfide scavenges the cytotoxic lipid oxidation product 4-HNE. *Neurotox Res* 17, 249–256 (2010).
129. Haouzi, P. *et al*. H2S induced hypometabolism in mice is missing in sedated sheep. *Respiratory Physiology & Neurobiology* 160, 109–115 (2008).
130. Drabek, T. *et al*. Intravenous hydrogen sulfide does not induce hypothermia or improve survival from hemorrhagic shock in pigs. *Shock* 35, 67–73 (2011).
131. Simon, F. *et al*. Hemodynamic and metabolic effects of hydrogen sulfide during porcine ischemia/reperfusion injury. *Shock* 30, 359–364 (2008).
132. Yong, R. & Searcy, D. G. Sulfide oxidation coupled to ATP synthesis in chicken liver mitochondria. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol*. 129, 129–137 (2001).
133. Wang, R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol. Rev*. 92, 791–896 (2012).
134. Wang, M.-J. *et al*. The hydrogen sulfide donor NaHS promotes angiogenesis in a rat model of hind limb ischemia. *Antioxid Redox Signal* 12, 1065–1077 (2010).
135. Gozzelino, R., Jeney, V. & Soares, M. P. Mechanisms of cell protection by heme oxygenase-1. *Annu. Rev. Pharmacol. Toxicol*. 50, 323–354 (2010).
136. Zuidema, M. Y., Peyton, K. J., Fay, W. P., Durante, W. & Korthuis, R. J. Antecedent hydrogen sulfide elicits an anti-inflammatory phenotype in postischemic murine small intestine: role of heme oxygenase-1. *Am J Physiol Heart Circ Physiol* 301, H888–94 (2011).
137. Taipale, M., Jarosz, D. F. & Lindquist, S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat Rev Mol Cell Biol* 11, 515–528 (2010).
138. Meng, J.-L. *et al*. Heat shock protein 90 mediates cytoprotection by H_2S against chemical hypoxia-induced injury in PC12 cells. *Clin Exp Pharmacol Physiol* 38, 42–49 (2011).
139. Olson, K. R. A practical look at the chemistry and biology of hydrogen sulfide. *Antioxid Redox Signal* 17, 32–44 (2012).
140. Olson, K. R. Hydrogen sulfide: both feet on the gas and none on the brake? *Front Physiol* 4, 2 (2013).
141. Mathai, J. C. *et al*. No facilitator required for membrane transport of hydrogen sulfide. *Proc Natl Acad Sci USA* 106, 16633–16638 (2009).
142. Chen, Y. *et al*. A Ratiometric Fluorescent Probe for Rapid Detection of Hydrogen Sulfide in Mitochondria. *Angew. Chem. Int. Ed. Engl*. (2013). doi:10.1002/anie.201207701
143. Derwall, M. *et al*. Hydrogen sulfide does not increase resuscitability in a porcine model of prolonged cardiac arrest. *Shock* 34, 190–195 (2010).
144. Pan, T.-T., Chen, Y.Q. & Bian, J.-S. All in the timing: a comparison between the cardioprotection induced by H2S preconditioning and post-infarction treatment. *Eur. J. Pharmacol*. 616, 160–165 (2009).
145. Moers, C. *et al*. Machine Perfusion or Cold Storage in Deceased-Donor Kidney Transplantation. *N Engl J Med* 360, 7–19 (2009).
146. Moers, C., Pirenne, J., Paul, A., Ploeg, R. J. Machine Preservation Trial Study Group. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med* 366, 770–771 (2012).

2

HYDROGEN SULFIDE-INDUCED HYPOMETABOLISM PREVENTS RENAL ISCHEMIA / REPERFUSION INJURY IN MICE

EM Bos
HGD Leuvenink
PM Snijder
NJ Kloosterhuis
JL Hillebrands
JC Leemans
S Florquin
H van Goor

Published in the Journal of the American Society of Nephrology

Reference: J Am Soc Nephrol, 2009. Sept;20(9):1901-1905
Digital object identifier (DOI): 10.1681/ASN.2008121269

ABSTRACT

Hydrogen sulfide (H_2S) has been historically perceived as a dangerous and toxic gas until the recent discovery that this substance can induce a hypometabolic, hibernation-like state in mammals when given in subtoxic concentrations. Reducing the demand for oxygen using pharmacological means is a novel and promising strategy for minimizing hypoxia-induced injury, such as the ischemia/reperfusion injury that is unavoidable during the renal transplant process. We show that H_2S can reduce metabolism in-vivo, ex-vivo and in-vitro. Furthermore, we demonstrate the beneficial effects of H_2S -induced hypometabolism in a model of bilateral renal ischemia/reperfusion injury using three different treatment strategies. The results demonstrate striking protective effects on survival, renal function, apoptosis and inflammation and indicate that a state of reduced metabolism induced by H_2S might be valuable in protecting kidneys that suffer from hypoxia.

INTRODUCTION, RESULTS AND DISCUSSION

The toxicity of H_2S has long been studied because of its involvement in deadly industrial and agricultural accidents[1]. Recently, an unknown property of the gas was revealed, namely the ability to induce hypometabolism in naturally non-hibernating mammals[2,3]. Mice exposed to subtoxic concentrations of gaseous H_2S rapidly and reversibly enter a hibernation-like state. During H_2S -treatment, metabolic parameters rapidly decrease: a ~60% reduction in CO_2-production and O_2-consumption within minutes of exposure (Figure 1A), which can decline even further to more than 90%[2-4]. In addition, the core body temperature decreases to near-ambient temperature and heart rate and breathing frequency are significantly lower[2]. The demand for oxygen is reduced to such an extent that H_2S -treated mice can survive in 5% oxygen for over 6 hours, while untreated controls die within 15 minutes[4]. In-vitro, H_2S can reversibly reduce mitochondrial oxygen consumption[5,6] and mitochondrial membrane potential (Figure 1B). Ex-vivo H_2S can reduce O_2-consumption and total ATP content of the isolated perfused kidney (Figure 1C, D). H_2S also has antioxidant capacity, either by direct scavenging of reactive oxygen or nitrogen species or indirectly by increasing cellular glutathione levels[3]. We hypothesized that a state of extremely low metabolism induced by exposure to gaseous H_2S would provide protection during periods of ischemia and reperfusion by reducing the demand for oxygen.

To investigate the protective potential of H_2S -induced hypometabolism we used a model of bilateral renal ischemia/reperfusion in the mouse. We evaluated four different treatment regimens (Figure 1E), comparing pretreatment, posttreatment and pre- and posttreatment with 100 ppm H_2S (n=6-7 per group). In both pretreated groups, C57BL/6 mice were first treated with H_2S for 30 minutes to induce hypometabolism. Our initial experiments showed that the induction of hypometabolism typically takes place within the first 10 minutes of exposure (Figure 1A). After the pretreatment period, renal blood flow was interrupted for 30 minutes by placing non-traumatic vascular clamps over both renal pedicles. To study the effects of H_2S on reperfusion damage alone, posttreatment with H_2S started 5 minutes before removal of the clamps, and lasted for 35 minutes. The pre- and posttreatment group received H_2S starting 30 minutes before ischemia until 30 minutes after reperfusion. To separate the effects of H_2S from the already well known protective effects of hypothermia, core body temperature of all animals was maintained at 37°C during and after the procedure. Induction of hypometabolism in H_2S treated animals was confirmed by lowered breathing frequency and CO_2-production, measured using closed system respirometry (Figure 1F, G).

Bilateral ischemia caused excessive renal damage in the control group, leading to an impaired 3-day survival caused by renal failure (Figure 2A). Both groups in which mice were pretreated with H_2S had 100% survival after three days ($p<0.001$), while mice that only received H_2S during reperfusion showed similar survival to the control group (p=ns). Serum creatinine and urea measurements were performed to quantify the renal function loss associated with bilateral renal ischemia/reperfusion. Control and posttreatment animals showed highly elevated levels of both creatinine and urea (Figure 2B, Supplementary Figure 1), while animals pretreated with H_2S had only slightly higher levels than sham operated animals (p=ns). These

Figure 1

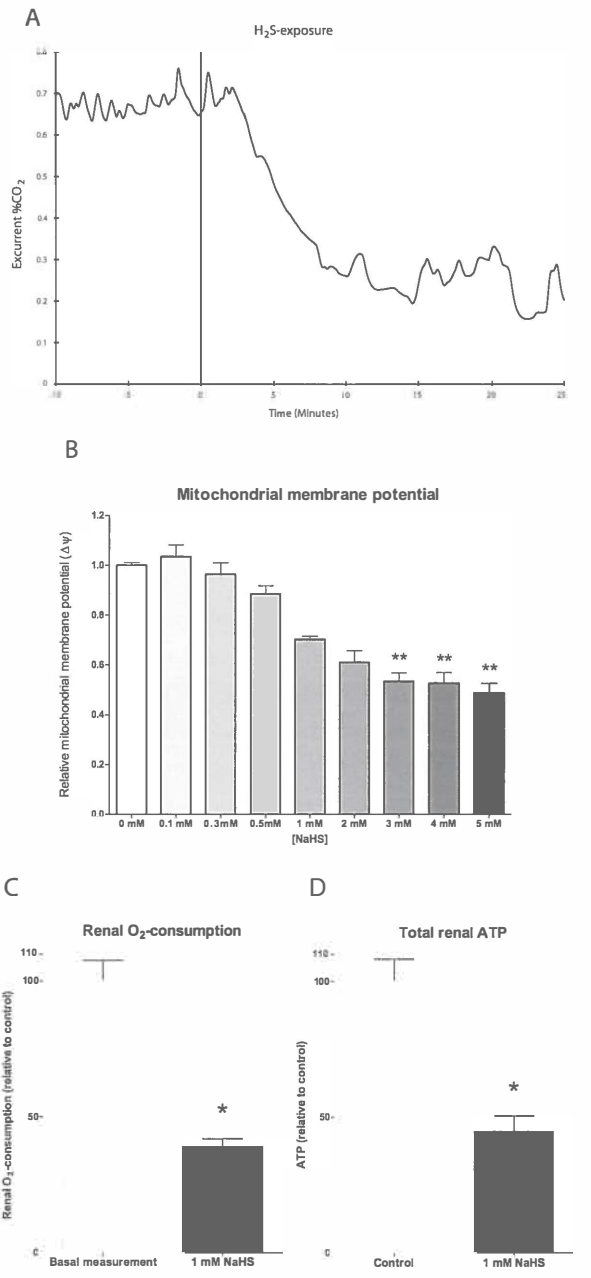


Figure 1 – Metabolic suppression by H_2S and experimental design.
(A) Exposure to 100 ppm H_2S causes a rapid reduction in CO_2-production of a single mouse. (B) NRK-52E proximal tubular cells loaded with the mitochondrial membrane potential indicating fluorescent dye JC-1 were exposed to different concentrations of NaHS (a donor of H_2S in solution) for 20 minutes. **P < 0.01 (C, D) Rat kidneys in an isolated perfused kidney setup were exposed to 1 mM NaHS (n=4) for 30 minutes, and O_2-consumption and ATP were compared to controls (n=3). *P < 0.05.

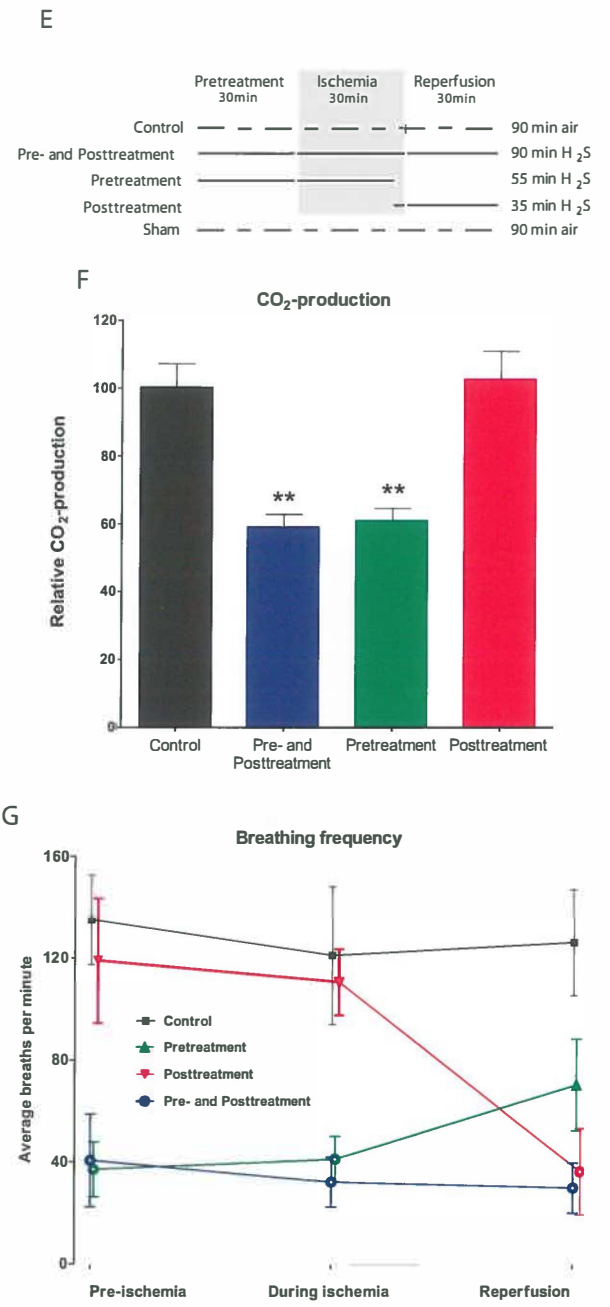


Figure 1 [Continued]
(E) Schematic of experimental design showing different H_2S treatment regimens. (F) Relative CO_2-production of animals during the period of ischemia, corrected for body weight (n=7). **P < 0.01 (G) Average breathing frequency of animals 5 minutes before ischemia, during ischemia, and 30 minutes after ischemia (n=5). Open circles indicate periods where animals received 100 ppm H_2S.

Figure 2

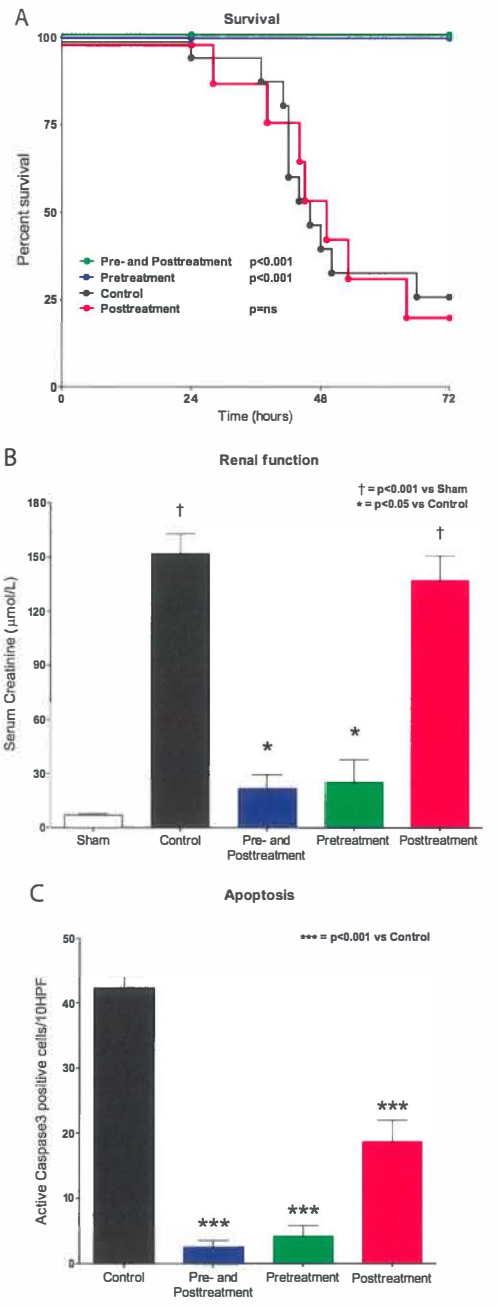


Figure 2 – H_2S -induced hypometabolism prevents mortality and renal damage after renal ischemia. (A) Three-day survival of animals after reperfusion. (B) Renal function as measured by serum creatinine after 1 day of reperfusion. *p<0.05 vs. Control, †p<0.001 vs. Sham (C) Apoptosis after 1 day of reperfusion was scored in sections stained for active Caspase 3 using immunohistochemistry. Apoptotic tubular cells were counted at 400x magnification in 10 non-overlapping fields. ***P < 0.001.

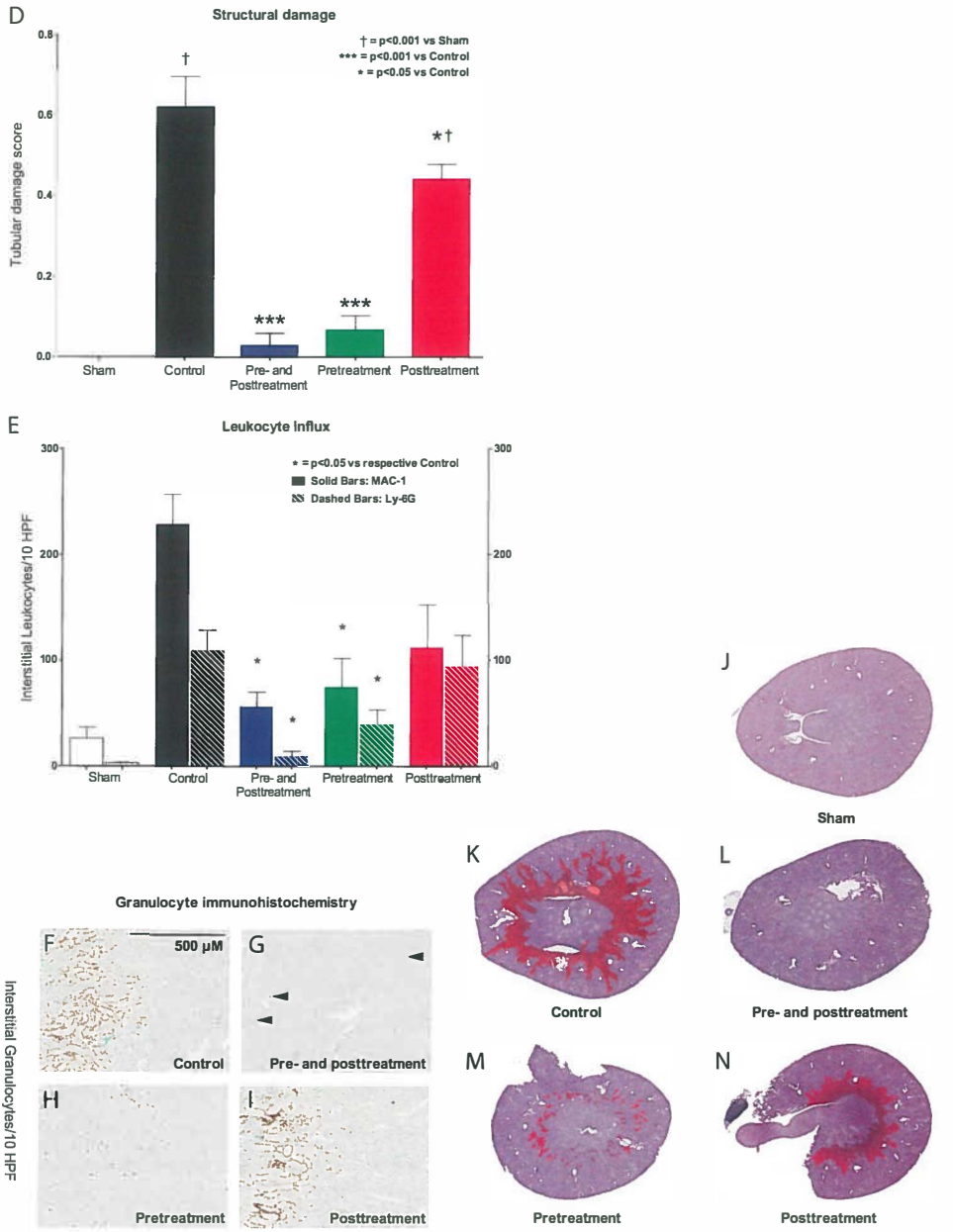


Figure 2 [Continued]

(D) Structural damage as assessed in PAS-stained sections after 1 day of reperfusion. *P < 0.05 vs. Control, ***P < 0.001 vs. Control, †p<0.001 vs. Sham (E) Influx of leukocytes and granulocytes into the renal interstitium was scored in sections stained for MAC-1 (solid bars) or Ly-6G (dashed bars) using immunohistochemistry. *P < 0.05 vs. Control (F-I) Representative photomicrographs of Ly-6G stained sections. (J-N) Representative PAS-stained renal sections with necrotic area artificially colored red, indicating the extent of necrotic damage found in each group. (For B, C, D, E: Sham (n=5), Control (n=7), H_2S -treated groups (n=6)).

measurements indicate massive renal failure in the control and posttreatment groups, which is the most likely cause of the diminished survival in these groups.

We assessed structural renal damage in Periodic acid-Schiff (PAS) stained sections and found a similar pattern to the renal function measurements as expected. Massive acute tubular necrosis was detected in control animals at day 1, whereas mice in both pretreated groups had no or minimal renal damage (Figure 2D, J-N, Supplementary Figure 3). Posttreatment with H_2S showed a significant reduction in tubular damage compared to controls, although it was not as extensive as in pretreated animals. After 3 days, a similar pattern was seen (Supplementary Figure 2). Posttreatment did not have significant protective effects at this time point, although these results are confounded to some extent, since animals with large amounts of renal damage had already deceased at this point.

Active Caspase 3 staining using immunohistochemistry indicated that IRI-induced apoptosis is also prevented by H_2S-pretreatment. (Figure 2C, Supplementary Figure 4). A less pronounced but statistically significant effect was seen in the Posttreatment group. Real-Time PCR measurements showed that mRNA expression of pro-apoptotic BAX was 2.5 times higher in control kidneys compared to sham operated animals (Supplementary Figure 5A). Expression was not significantly increased in animals pretreated with H_2S. The expression of anti-apoptotic BCL-2 did not differ between groups (Supplementary Figure 5B) indicating that the anti-apoptotic effects of H_2S are not mediated through induction of BCL-2 mRNA expression. Whether H_2S directly or indirectly inhibits increased expression of BAX is not clear. Transmission electron microscopy of a limited number of samples implies that H_2S treatment protected against loss of mitochondrial integrity and mitochondrial swelling (Supplementary Figure 6). In literature, pro-apoptotic as well as anti-apoptotic effects of H_2S are described[5;7-9], and it is not known whether H_2S can directly modulate apoptotic pathways, or that increased mitochondrial integrity and reduced mitochondrial stress caused by reduced mitochondrial activity caused the reduction in Caspase 3 activity in the posttreatment group.

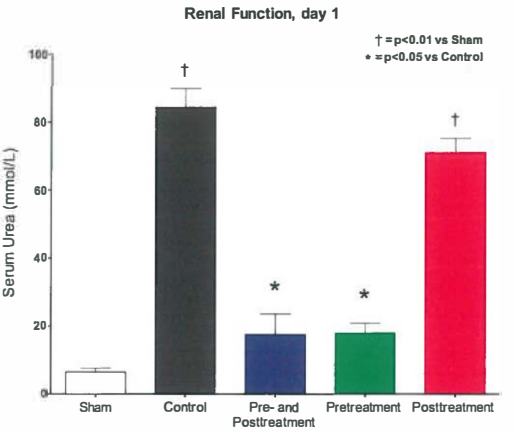
We studied the inflammatory component of ischemia / reperfusion injury by immunohistochemical staining for MAC-1 (CD11b, which is present on macrophages, monocytes, granulocytes and natural killer cells[10]) and Ly-6G (which is expressed on mature granulocytes). (Figure 2E-I, Supplementary Figure 7). The influx of MAC-1 and Ly-6G positive cells was greatly reduced by H_2S -pretreatment ($p<0.05$), but was not significantly affected by posttreatment.

These results indicate that the reduction in metabolism prior to ischemia is highly protective in reducing ischemia-induced injury with predictable onset, such as during transplantation or surgical intervention. The mechanism of H_2S-induced hypometabolism is unknown as of yet, but is most likely mediated through reversible inhibition of complex IV (cytochrome oxidase)[11], the terminal enzyme of the mitochondrial electron transport chain. Inhibition of this complex might be the mechanism of the reduction in mitochondrial membrane potential caused by H_2S-treatment. It seems unlikely that H_2S directly and effectively inhibits necrotic-, apoptotic- and inflammatory pathways after an ischemic insult. The observation that protection is greatest when H_2S is given before and during, but much less when given directly after the hypoxic period, supports the notion that the reduction in oxygen demand during hypoxia prevents the activation of these detrimental pathways. The moderate effects of H_2S in the posttreatment

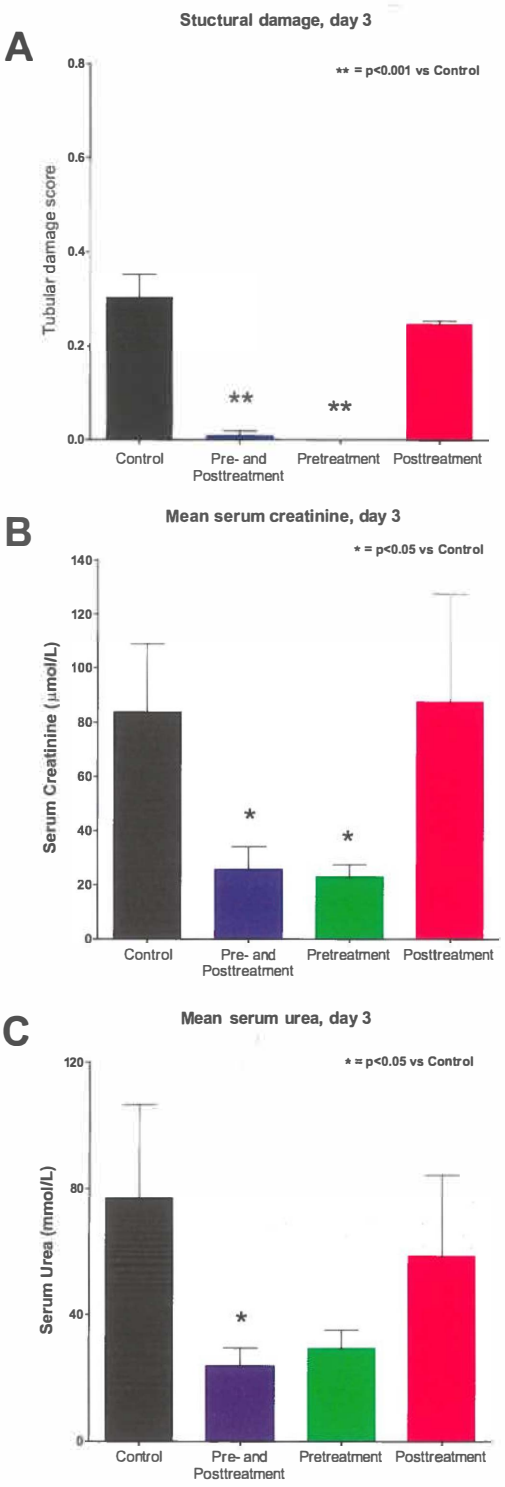
group could be caused by the inhibition of ROS production by decreasing mitochondrial activity. Protection could also be mediated through direct antioxidative action[3] or increased glutathione levels caused by H_2S[3].

Recent literature shows beneficial effects of gaseous H_2S on survival in models of hypoxia[4] and hemorrhagic shock[12]. Other groups have studied the protective effects of soluble forms of H_2S (such as NaHS or Na_2S) in models of ischemia. These studies show beneficial effects of H_2S on renal-[13], cardiac-[5], hepatic-[14] and pulmonary ischemia[15]. One paper suggests an association between H_2S treatment and reduced activation of multiple signal transduction molecules, such as p38, ERK and JNK. However, a direct relationship between H_2S and kinase activation was not proven. We found that phosphorylation of ERK1/2 was stimulated by ischemia in our model, but no modulation was seen in H_2S -treated animals (Supplementary Figure 8). Our study shows a novel relation between H_2S treatment and hypometabolism, which has not been previously investigated. The protective effects of H_2S -treatment post-hypoxia are less pronounced in our experiments. However, a recent paper indicated that injection of Na_2S just prior to reperfusion in a model of myocardial infarction caused a great reduction in infarct size and protected mitochondrial integrity and function[5]. This indicates that posttreatment with H_2S might still be a promising intervention in cutting back on the detrimental effects of hypoxia after the event. We conclude that hypometabolism induced by gaseous H_2S is a novel treatment regimen with high therapeutic potential in reducing renal damage associated with ischemic insults.

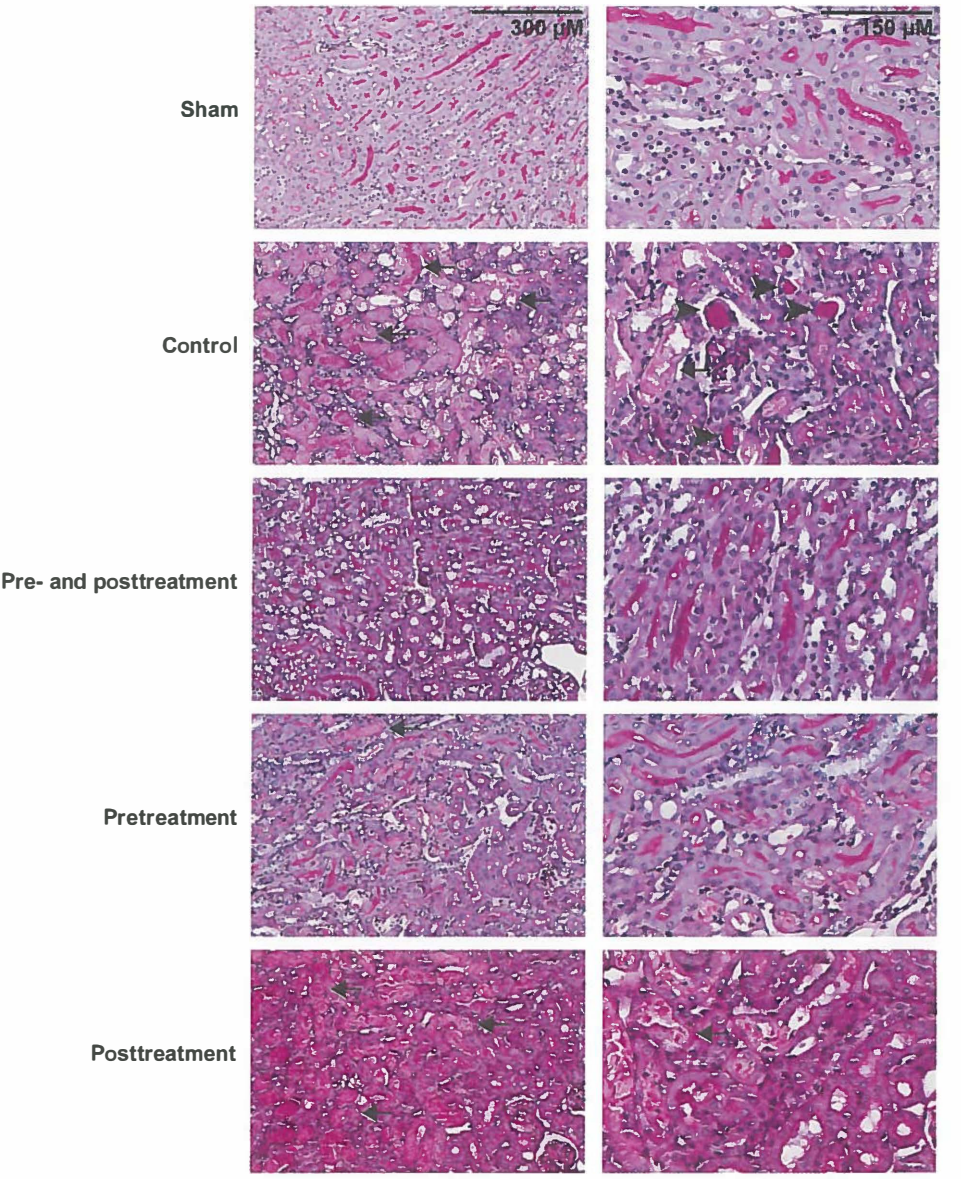
SUPPLEMENTARY FIGURES



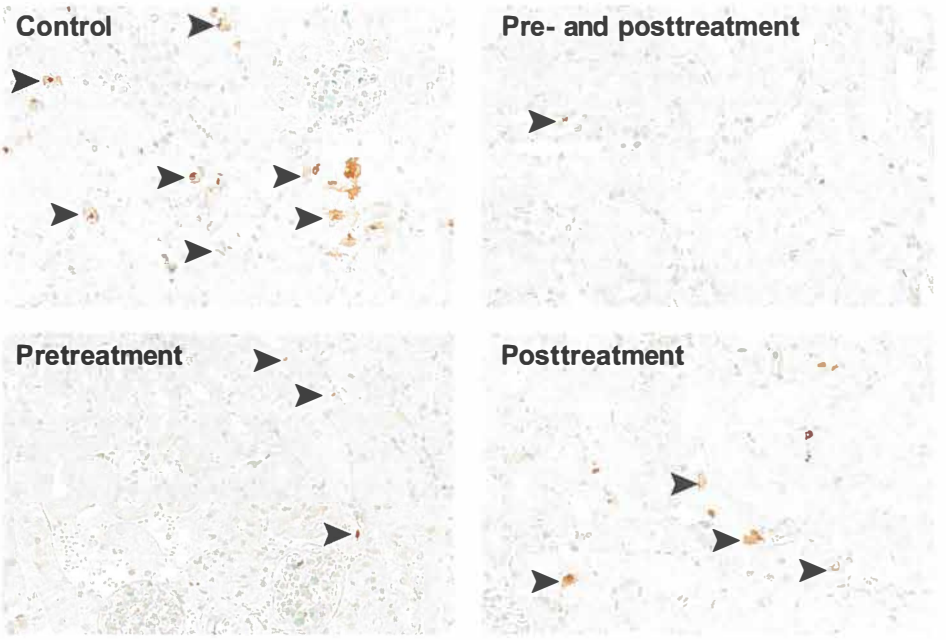
Supplementary Figure 1 – Renal function as measured by serum urea after 1 day of reperfusion.
*p<0.05 vs. Control, †p<0.01 vs. Sham.



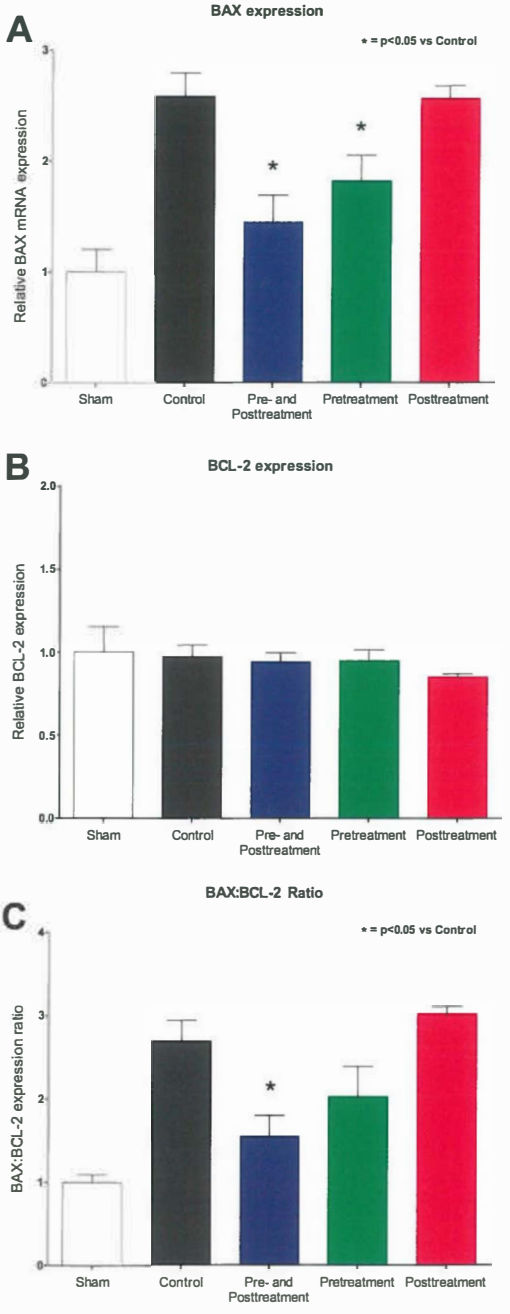
Supplementary Figure 2
(A) Structural damage as assessed in PAS-stained sections after 3 days of reperfusion. **p<0.001 vs. Control. (B) Renal function as measured by serum creatinine and (C) serum urea after 3 days of reperfusion. *p<0.05 vs. Control.



Supplementary Figure 3 – Representative photomicrographs of PAS-stained renal tissue from all groups after 1 day of reperfusion.
Sham kidneys show normal morphology, while control kidneys show necrosis (arrows) and cast deposition (arrowheads). Normal morphology is seen in kidneys from the pre- and posttreatment group. Kidneys from the pretreatment group show only minimal necrosis, while posttreatment kidneys show intermediate amounts of damage.

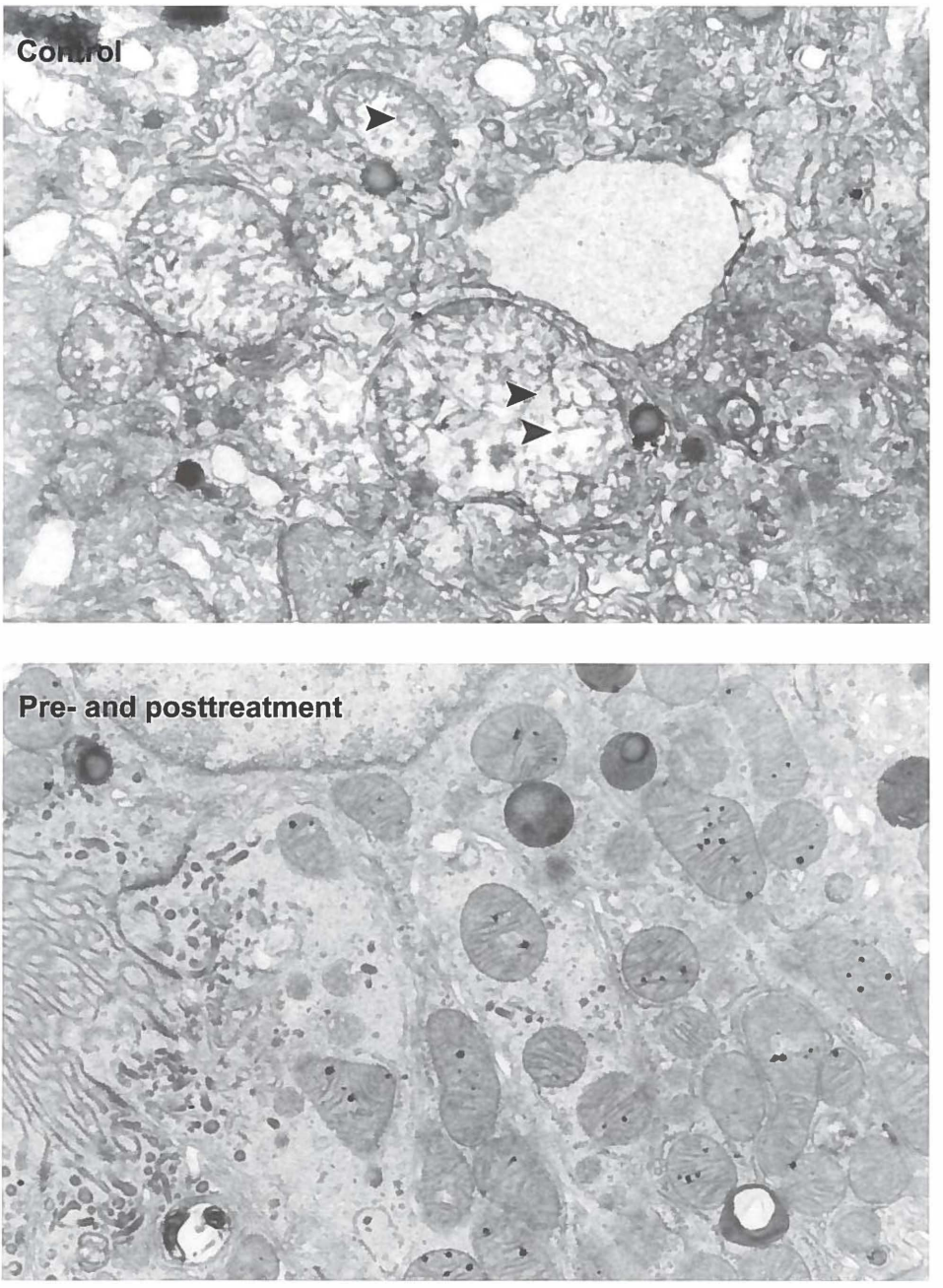


Supplementary Figure 4 – Representative photomicrographs of renal tissue stained for active Caspase 3, showing greatly reduced numbers of active Caspase 3 positive tubular cells in all treated groups.
Arrowheads indicate active Caspase 3 positive cells.

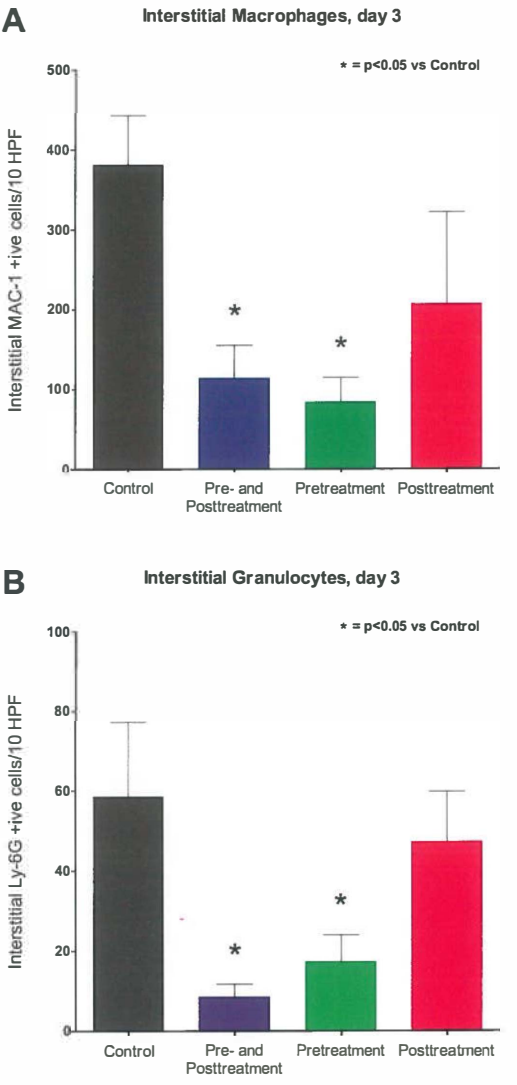


Supplementary Figure 5

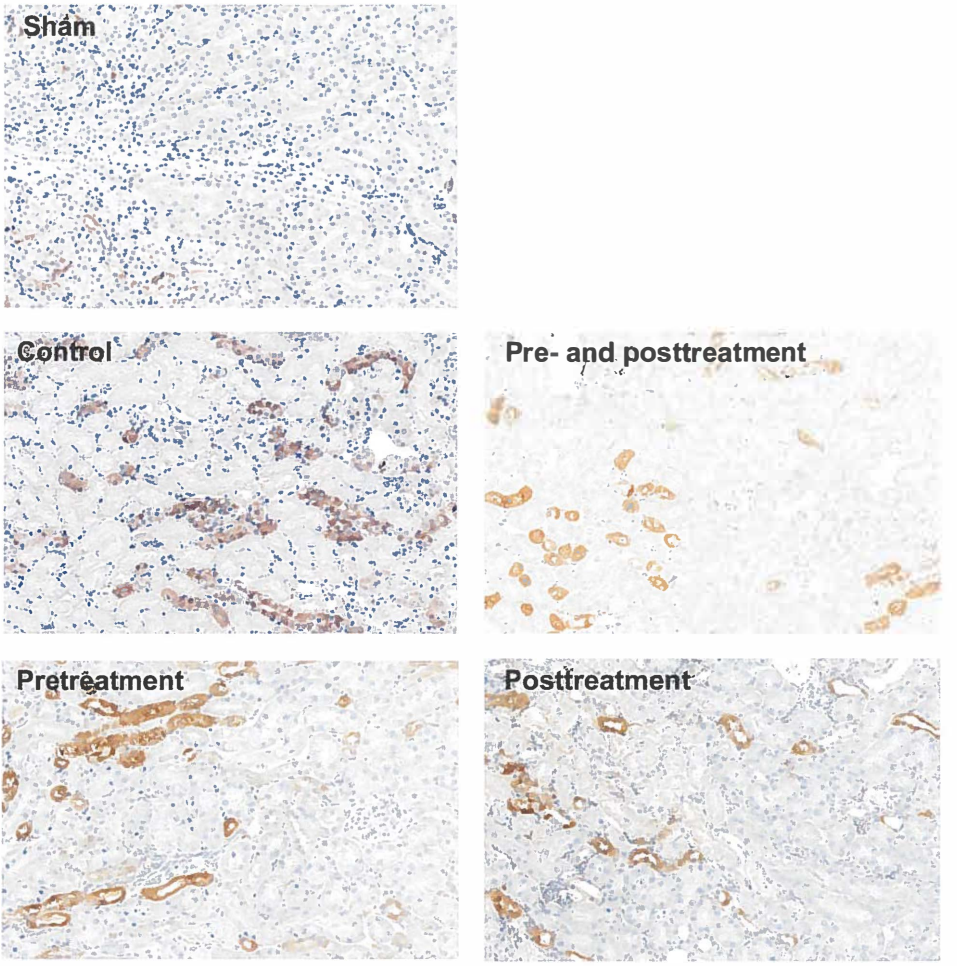
(A) BAX mRNA expression and (B) BCL-2 mRNA expression after 1 day of reperfusion as assessed by quantitative Real-Time PCR. (C) The ratio of the pro-apoptotic BAX vs. anti-apoptotic BCL-2 mRNA expression. *p<0.05.



Supplementary Figure 6 – Transmission electron microscopy images of a tubule in a control and a pre-and posttreated kidney.
The controls showed large, swollen mitochondria, loss of mitochondrial integrity and loss of mitochondrial crypts (arrowheads). Pre- and posttreatment kidneys showed normal mitochondrial morphology.



Supplementary Figure 7
(A) Interstitial macrophages and (B) interstitial granulocytes after three days of reperfusion in kidney sections stained using immunohistochemistry. *p<0.05 vs. Control.



Supplementary Figure 8 – Representative photomicrographs of renal sections from all treated groups, stained for phosphorylated ERK1/2 (pERK1/2) using immunohistochemistry.
Increased activation compared to sham levels is seen in all groups exposed to IRI, however, no modulation by H_2S treatment could be detected.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (6-8 week old, Harlan, Zeist, the Netherlands) and male inbred Fisher F344 rats (250-300 gram, Harlan, Zeist, the Netherlands) were housed under standard conditions with a 12h light:dark cycle at our animal research facility with ad libitum access to water and murine chow. Experimental procedures were in agreement with institutional and legislator regulations and approved by the local committee for animal experiments.

Respirometry

Measurement of animal CO_2-production and O_2-consumption was performed using an advanced, modular respirometry system (TR-3 system, Sable Systems, Las Vegas, NV, USA). Air or H_2S /Air mixture was pushed through a mass flow controller (Model 840, Sierra Instruments, Egmond, the Netherlands) set to 200 mL/min. Animals were placed in a downstream, airtight respirometry chamber with heat pads. The excurrent gas was subsequently lead through a CA-10a dual wavelength infrared sensor CO_2-analyzer (Sable Systems) and a custom-made PA-10a paramagnetic O_2-analyzer with stainless steel inner tubing (Sable Systems). Data acquisition was performed using a UI-2 interface and ExpeData v1.0.24 software (Sable Systems). Compressed air and 500 ppm H_2S / N_2 (Air Products, Amsterdam, the Netherlands) were mixed in a 4:1 ratio, producing a 100 ppm H_2S /17% O_2 mixture. Control animals received room air. CO_2-production was corrected for body weight and normalized to mean control values.

Renal ischemia/reperfusion

Both renal pedicles were clamped for 30 minutes using non-traumatic vascular clamps through a midline abdominal incision under general anesthesia (0.07 ml/10 g mouse of 1.25 mg/ml midazolam (Roche Diagnostics Corp), 0.08 mg/ml fentanyl citrate, and 2.5 mg/ml fluanisone (Janssen Pharmaceutica)). After removing the clamps, kidneys were inspected for restoration of blood flow and the muscle and skin layers were sutured with 5-0 stitches. Body temperature was monitored with a rectal probe and maintained at 37°C using heat pads and lamps. Sham operated animals received the exact same procedure, excluding the placement of the clamps on the renal pedicles. Breathing frequency was regularly counted. Subsequent to closure of the abdomen, all mice received a subcutaneous injection of 50 µg/kg buprenorphin (Schering-Plough) for analgesic purposes and were allowed to recover from surgery at 35°C in a ventilated incubator. Mice were sacrificed 24 and 72 hours after reperfusion. At the time of sacrifice mice were anesthetized using 2,5% isoflurane in O_2, blood was collected in heparin containing tubes and stored at -80°C, and kidneys were perfused with saline and harvested. A midcoronal kidney slice was fixed in 4% paraformaldehyde, processed for paraffin embedding and used for immunohistochemical analysis.

Plasma biochemical analysis

Renal function was determined by measuring creatinine and urea in plasma samples using standard methods by our hospital research services.

Histopathological scoring

The extent of tubulo-interstitial damage was determined in Period Acid-Schiff (PAS)-stained sections, which were blindly scored. The ratio of necrotic cortical tubule surface area to total cortical surface area was determined at a 100x magnification. The necrotic area calculated in this manner correlated linearly with serum creatinine with an R^2 of 0,7677 and with serum urea with an R^2 of 0,6997.

Immunohistochemistry for Caspase 3, Ly-6G and pERK1/2

Immunohistochemical staining for active Caspase 3 and granulocytes was performed as described before16. In short, for active Caspase 3, paraffin embedded sections were stained using rabbit anti-human active Caspase 3 polyclonal antibody (Cell Signaling), followed by HRP-conjugated goat-anti-rabbit IgG (Immunovision Technologies). Positive tubular epithelial cells were counted in 10 non-overlapping fields at 400x magnification. For granulocytes, paraffin embedded sections were stained for Ly-6G using rat-anti-mouse Ly6G/C-FITC IgG2b antibody, followed by rabbit-anti-FITC and HRP-conjugated goat-anti-rabbit antibodies. Positive interstitial granulocytes were counted in 30 non-overlapping fields at 400x magnification. For phosphorylated ERK1/2, paraffin embedded sections were stained using Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) rabbit monoclonal antibody (Cell Signaling) followed by HRP-conjugated goat-anti-rabbit IgG and HRP-conjugated rabbit-anti-goat IgG antibodies (DAKO, Glostrup, Denmark).

Macrophage immunohistochemistry

Immunohistochemical staining for macrophages was performed on 4 µm cryosections using anti MAC-1 antibody (ab6332, Abcam, Cambridge, MA, USA) incubated for 1 hour at 1:250 dilution, followed by HRP-conjugated rabbit-anti-rat and subsequently HRP-conjugated goat-anti-rabbit antibodies. Positive interstitial macrophages were counted in 30 non-overlapping fields at 400x magnification.

Qualitative Real-Time Polymerase Chain Reaction

RNA was extracted from frozen human kidney biopsies using the TRIZOL method (Invitrogen, Carlsbad, CA). DNAse treatment was performed using Turbo DNAse-free (Ambion, Austin, TX). cDNA was synthesized using Superscript II RT and random hexamer primers (Invitrogen). A relative quantification PCR was performed to determine gene expression (Applied Biosystems, Foster City, CA). GAPDH was used as housekeeping gene. We used gene expression assays from Applied Biosystems:

BAX: Mm00432050_m1
BCL-2: Mm00477631_m1
GAPDH: Mm99999915_g1.

PCR was performed in a total volume of 20 µl containing 10ng cDNA template and 10 µl PCR-mastermix (Eurogentec). The Thermal Profile was 15 minutes at 95ºC, followed by 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. The average Ct-values for the target genes were subtracted from the average GAPDH Ct-values to yield the delta Ct. Results were expressed as $2^{-\Delta Ct,}$ normalized to mean sham values.

Cell culture

The normal rat kidney-52E (NRK-52E, ATCC) cell line is an immortalized line featuring the characteristics of rat renal proximal tubular cells. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Paisley, UK) containing 4,5 g/l glucose, 5% fetal calf serum (FCS; Bodinco, Alkmaar, the Netherlands), penicillin (100 U/mL) streptomycin (100 µg/mL). Cells were cultured using 75 cm2 collagen coated flasks (Corning, Schiphol-Rijk, Netherlands) in a humidified atmosphere of 5% CO_2 at 37°C.

JC-1 measurements

Cytofluometric analysis of mitochondrial membrane potential was performed by using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1, Invitrogen, Leek, Netherlands). JC-1 penetrates the cytosol of eukaryotic cells and exhibits potential-dependent accumulation specifically in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Cells grown to 80-90% confluence were harvested and seeded in a 24-well plate at a density of ~10.000 cells/well in 500 µL medium. After overnight culture, cells were washed with PBS and incubated with JC-1 in serum-free DMEM for 15 minutes at 37°C in the dark with 15 µg/ml JC-1. After incubation, cells were washed three times with PBS and medium without FCS was added. A baseline measurement of the fluorescence was performed using a spectrophotometer (Victor 3, PerkinElmer, Groningen, the Netherlands) at 485 nm excitation/535 nm emission and at 530 nm excitation/590 nm emission. Different concentrations of NaHS (Sigma-Aldrich, the Netherlands) were added to the medium and fluorescence was remeasured after 20 minutes. Data were corrected for background fluorescence by subtracting values measured in cells not loaded with JC-1, and the change in of 590 nm to 535 nm fluorescence ratio compared to untreated cells was calculated.

Isolated Perfused Kidney (IPK)

The IPK setup used was identical to the one described previously by our group[17]. Rats were anesthetized using 2,5% isoflurane / O_2 and cannulas were placed in the renal artery, renal vein and ureter. After placement in the IPK-setup, the kidney was continuously perfused via the renal artery with warmed (37°C) and oxygenated (95% O_2 and 5% CO_2 gas mixture) Krebs-Henseleit-bicarbonate (KHB) solution at a pH of 7.4 ± 0.05 and a pO_2 > 100 kPa, by using a roller pump (Ismatec mv-ca/04; Ismatec, Glattbrugg, Switzerland) delivering a constant flow of 8 mL/min throughout the experiment. The composition of the KHB was as follows: 118 mM NaCl; 4.7 mM KCl; 2.5 mM CaCl2; 1.2 mM KH2PO4; 1.2 mM MgSO4; 25 mM NaHCO3; 10 mM glucose. After connecting the kidney, flow was gradually increased to 8 mL/min. Vascular responses were monitored by an electromechanical pressure transducer (Cobe; Arvada, CO) connected to a computer program (LabView, National Instruments, Austin, TX). After an equilibration period of 25 to 30 min, when renal vascular resistance had stabilized, pO_2 was measured using an ABL700 blood gas analyzer (Radiometer Medical, Denmark) in pre- and postrenal samples taken from an injection port 2 cm before the kidney and from the renal vein, respectively. After

these samples were taken, NaHS (Sigma Aldrich, the Netherlands) was added to the perfusate to reach a final concentration of 500 μM NaHS in the H_2S -treated group. After 30 minutes of NaHS perfusion, pO_2 measurements were taken, and cortical tissue was removed an snap-frozen in liquid N_2 for ATP measurements.

ATP measurement

Measurement of ATP was performed using the ATP Bioluminescence CLS-II kit (Roche Applied Science, Almere, the Netherlands) according to the manufacturers' protocol. ATP levels were corrected for total protein using the Lowry protein measurement system (Bio-Rad, Hercules, CA, USA)

Transmission Electron Microscopy

Investigation of mitochondrial structure and integrity was performed according to standard procedures on formalin/glutaraldehyde fixed renal samples.

Statistical analysis

Data were analyzed using GraphPad PRISM 5.0 (GraphPad, San Diego, USA) or SPSS 14.0 (SPSS inc., Chicago, IL, USA) using Mann-Whitney U tests, One-Way ANOVA or Kruskall Wallis tests where appropriate. Bonferroni or Dunns postcorrection was applied where multiple comparisons were made. Normality was tested using the Kolmogorov–Smirno test. For the histopathological scoring, both kidneys of each animal were analyzed and a mixed-effects model was used taking the animal of origin into account. $p<0.05$ was considered statistically significant. All data are expressed as the mean ± SEM (Standard Error of the Mean) unless otherwise indicated.

ACKNOWLEDGEMENTS

This study was supported by grants from the Jan Kornelis de Cock-foundation, the Groningen University Institute for Drug Exploration (GUIDE) and Carburos Metálicos S.A.

The authors would like to express their gratitude towards Marian Bulthuis, Michel Weij, Sippie Huitema, Pieter A. Klok, W. Geert van Rijt, Jacco J. Zwaagstra, Ruby D. Kalicharan and Loes Butter for their excellent technical assistance. We also thank John R.B. Lighton, Barbara Joos and Robbin J. Turner for their valuable help with the respirometry experiments.

REFERENCES

1. Reiffenstein RJ, Hulbert WC, Roth SH: Toxicology of hydrogen sulfide. Annu Rev Pharmacol Toxicol 32:109-134, 1992
2. Blackstone E, Morrison M, Roth MB: H_2S induces a suspended animation-like state in mice. Science 308:518, 2005
3. Szabó C : Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov 6:917-935, 2007
4. Blackstone E , Roth MB: Suspended animation-like state protects mice from lethal hypoxia. Shock 27:370-372, 2007
5. Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L, Jiao X, Scalia R, Kiss L, Szabo C, Kimura H, Chow CW, Lefer DJ: Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci U S A 104:15560-15565, 2007
6. Yong R, Searcy DG: Sulfide oxidation coupled to ATP synthesis in chicken liver mitochondria. Comp Biochem Physiol B Biochem Mol Biol 129:129-137, 2001
7. Yang G, Wu L, Wang R: Pro-apoptotic effect of endogenous H_2S on human aorta smooth muscle cells. FASEB J 20:553-555, 2006
8. Rinaldi L, Gobbi G, Pambianco M, Micheloni C, Mirandola P, Vitale M: Hydrogen sulfide prevents apoptosis of human PMN via inhibition of p38 and caspase 3. Lab Invest 86:391-397, 2006
9. Hu LF, Lu M, Wu ZY, Wong PT, Bian JS: Hydrogen sulfide inhibits rotenone-induced apoptosis via preservation of mitochondrial function. Mol Pharmacol 2008
10. Solovjov DA, Pluskota E, Plow EF: Distinct roles for the alpha and beta subunits in the functions of integrin alphaMbeta2. J Biol Chem 280:1336-1345, 2005
11. Khan AA, Schuler MM, Prior MG, Yong S, Coppock RW, Florence LZ, Lillie LE: Effects of hydrogen sulfide exposure on lung mitochondrial respiratory chain enzymes in rats. Toxicol Appl Pharmacol 103:482-490, 1990
12. Morrison ML, Blackwood JE, Lockett SL, Iwata A, Winn RK, Roth MB: Surviving blood loss using hydrogen sulfide. J Trauma 65:183-188, 2008
13. Tripatara P, Patel NS, Collino M, Gallicchio M, Kieswich J, Castiglia S, Benetti E, Stewart KN, Brown PA, Yaqoob MM, Fantozzi R, Thiemermann C: Generation of endogenous hydrogen sulfide by cystathionine gamma-lyase limits renal ischemia/reperfusion injury and dysfunction. Lab Invest 88:1038-1048, 2008
14. Jha S, Calvert JW, Duranski MR, Ramachandran A, Lefer DJ: Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. Am J Physiol Heart Circ Physiol 295:H801-H806, 2008
15. Fu Z, Liu X, Geng B, Fang L, Tang C: Hydrogen sulfide protects rat lung from ischemia-reperfusion injury. Life Sci 82:1196-1202, 2008
16. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der Poll T, Weening JJ, Florquin S: Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest 115:2894-2903, 2005
17. Maathuis MH, de Groot M, Ploeg RJ, Leuvenink HG: Deterioration of Endothelial and Smooth Muscle Cell Function in DCD Kidneys After Static Cold Storage in IGL-1 or UW. J Surg Res 152:231-7, 2009

3

BENEFICIAL EFFECTS OF GASEOUS HYDROGEN SULFIDE IN HEPATIC ISCHEMIA / REPERFUSION INJURY

EM Bos
PM Snijder
H Jekel
M Weij
JC Leemans
MCRF van Dijk
T Lisman
H van Goor
HGD Leuvenink

Published in Transplant International

Reference: Transplant Int, 2012. Jul;25(8):897-908
Digital object identifier (DOI): 10.1111/j.1432-2277.2012.01514.x

ABSTRACT

Hydrogen sulfide (H_2S) can induce a reversible hypometabolic state, which could protect against hypoxia. In this study we investigated whether H_2S could protect livers from ischemia/reperfusion injury (IRI).

Male C57BL/6 mice were subjected to partial hepatic IRI for 60 minutes. Animals received 0 (IRI) or 100 ppm H_2S (IRI + H_2S) from 30 minutes prior to ischemia until 5 min before reperfusion. Core body temperature was maintained at 37°C. Animals were sacrificed after 1, 6 or 24 hours.

Hepatic ischemia caused extensive hepatic necrosis in the IRI animals which coincided with an increase in ALT and AST serum levels. Animals treated with H_2S showed attenuated serum ALT and AST levels and reduced necrotic lesions after 24h. IRI animals had increased Bcl-2 mRNA expression and increased active Caspase 3 protein, which were both significantly lower in H_2S treated animals. Increased TNFα and IL-6 mRNA in the IRI livers was significantly attenuated by H_2S treatment, as was hepatic influx of Ly-6G positive granulocytes. Hepatic superoxide production after ischemia was attenuated by H_2S treatment.

In hepatic ischemia/reperfusion injury, gaseous H_2S treatment is highly protective, substantially reducing necrosis, apoptosis and inflammation. Gaseous H_2S is therefore a very promising treatment for reducing IRI during hepatic transplantation.

INTRODUCTION

Hepatic ischemia/reperfusion injury (IRI) is the result of transient blood flow deprivation, often seen during surgical intervention and liver transplantation. The detrimental effects of IRI have clinical consequences in the transplant setting, attributing to organ failure and rejection[1-3]. From animal experiments it has become clear that the hepatic response to IRI has different facets. In the early phase there is substantial production of reactive oxygen- and nitrogen species (ROS and RNS), causing necrosis and apoptosis[4,5]. In addition, the response to injury will result in upregulation of adhesion molecules, influx of leukocytes and further activation of Kupffer cells, which induces increased production of ROS and of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin 6 (IL-6)[5].

Hydrogen sulfide (H_2S), mostly known for its toxic properties[6], is a widely studied molecule. In recent years, however, H_2S has matured from a dangerous and malodorous gas to a physiologically important signaling molecule[7]. The functions of H_2S range from vasodilatation[8-11], inhibiting platelet aggregation[12], regulating bile excretion[13], stimulating angiogenesis[14] and neuromodulation[14-16] to scavenging of free radicals[17-19]. It is now, in addition to nitric oxide (NO) and carbon monoxide (CO), acknowledged as the third gasotransmitter, sharing many functions with these gases[20].

In addition to these physiological properties, one of the most captivating discoveries concerning H_2S in recent years is its ability to induce a reversible, hibernation-like state in naturally non-hibernating mammals[21]. Subtoxic concentrations of gaseous H_2S rapidly reduce metabolic parameters in mice, slowing down O_2 consumption and CO_2 production by ~60% within 5 minutes, and more than 90% after 6 hours of treatment. Core body temperature, heart rate and breathing frequency all decline to a great extent during treatment. When exposure to H_2S is stopped, animals recover rapidly without any apparent toxic effects. Although the mechanism behind H_2S -induced hypometabolism is unknown as of yet, one of the involved proteins likely to be involved is cytochrome c oxidase, the terminal enzyme in the mitochondrial electron transport chain. H_2S can reversibly inhibit mitochondrial oxygen consumption and ATP production by binding to cytochrome c oxidase[22,23]. In addition to protective effects of H_2S mediated ROS scavenging, this process lowers the demand for O_2 and prevents mitochondrial ROS production.

To pharmacologically reduce the demand for O_2 is an attractive strategy to attenuate the effects of IRI, such as during the unavoidable periods of hypoxia during liver transplantation. The liver is very susceptible to IRI, and can only be preserved outside of the body without major influence on long term outcome for up to 16 hours by using hypothermia and preservation solutions[24]. If metabolism in the liver can be reversibly lowered, the time the liver can be preserved might be increased, hepatic function after transplantation could be improved and acute and chronic rejection could be reduced. In this study, we therefore investigate whether hydrogen sulfide protects the liver from IRI in an experimental mouse model.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (6-8 week old, Harlan, Zeist, the Netherlands, n=5 per group) were housed under standard conditions with a 12h light:dark cycle at our animal research facility with ad libitum access to water and murine chow. Experimental procedures were in agreement with institutional and legislator regulations and approved by the local committee for animal experiments.

Respirometry

Measurement of animal CO_2-production was performed using a modular respirometry system (TR-3 system, Sable Systems, Las Vegas, NV, USA). Air or H_2S / Air mixture was pushed through a mass flow controller (Model 840, Sierra Instruments, Egmond, the Netherlands) set to 200 mL/min. Animals were placed in a downstream, airtight respirometry chamber with heat pads. The excurrent gas was lead through a CA-10a dual wavelength infrared sensor CO_2-analyzer (Sable Systems). Data acquisition was performed using a UI-2 interface and ExpeData v1.0.24 software (Sable Systems). Compressed air and 500 ppm H_2S / N_2 (Air Products, Amsterdam, the Netherlands) were mixed in a 4:1 ratio, producing a 100 ppm H_2S / 17% O_2 mixture. Control animals received room air. CO_2-production was corrected for body weight and normalized to mean control values.

H_2S treatment

Animals were treated with 100 ppm H_2S or air for 25 minutes in our respirometry system (as described above) to induce a hypometabolic state. They were then transferred to the operating table, where they received 100 ppm H_2S or air through a standard anesthesia cap during the procedure until 5 minutes before reperfusion. After that, all animals received only ambient air.

Hepatic ischemia/reperfusion

The left hepatic artery and portal vein were clamped for 60 minutes using non-traumatic vascular clamps through a midline abdominal incision under general anesthesia (75 mg/kg ketamine, 1 mg/kg domitor), causing ischemia in the median and left lateral hepatic lobes. After removing the clamps, the liver was inspected for restoration of blood flow and the muscle and skin layers were sutured with 5-0 stitches. Body temperature was monitored with a rectal probe and maintained at ~37ºC using a heating pad and lamps. Sham-operated animals were subjected to the same procedure, excluding the placement of the clamps. Subsequent to closure of the abdomen, all mice received a subcutaneous injection of 50 µg/kg buprenorphine (Schering-Plough) for analgesic purposes and were allowed to recover from surgery at 35ºC in a ventilated incubator. Mice were sacrificed 1, 6 or 24 hours after reperfusion. At the time of sacrifice mice were anesthetized using 2.5% isoflurane in O_2, blood was collected in EDTA containing tubes, centrifuged for 10 minutes at 1000 rcf and serum was collected and stored at -80°C. Livers were perfused with 0.9% NaCl solution and ischemic and non-ischemic lobes were collected. Half of each lobe was fixed in 4% paraformaldehyde, processed for paraffin embedding and used for immunohistochemical analysis. The other half was snap frozen in liquid nitrogen and stored at -80°C.

Dihydroethidine (superoxide) staining

Frozen liver sections (4 µm) were dried for 20 minutes under a room temperature blower and subsequently incubated for 30 minutes at 37°C with 12,5 µM dihydroethidine (Invitrogen) dissolved in PBS. Images were acquired at 20x magnification and analyzed using NCBI ImageJ.

Plasma biochemical analysis

Hepatic damage was assessed by measuring aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) in plasma samples using standard methods by our hospital research services.

Histopathological scoring

The extent of hepatocellular damage and necrosis was determined in haematoxylin-eosin (HE) stained sections. Whole slides were scanned using an Aperio ScanScope GS (Aperio Technologies, Vista, CA, USA). Total hepatic area and necrotic hepatic area were determined using the Aperio Imagescope software, and the ratio of necrotic hepatic surface area to total hepatic surface area was determined. The necrotic area calculated in this manner correlated linearly with serum AST (24h) with an r^2 of 0,9252 and with serum ALT (24h) with an r^2 of 0,8792.

Immunohistochemistry for active Caspase 3 and Ly-6G

Immunohistochemical staining for active Caspase 3 and granulocytes was performed as described before[25]. In short, for active Caspase 3, paraffin embedded sections were stained using rabbit anti-human active Caspase 3 polyclonal antibody (Cell Signaling), followed by HRP-conjugated goat-anti-rabbit IgG (Immunovision Technologies). For granulocytes, paraffin embedded sections were stained for Ly-6G using rat-anti-mouse Ly6G / C-FITC IgG2bantibody (AbCam, Cambridge, MA, USA), followed by rabbit-anti-FITC and HRP-conjugated goat-anti-rabbit antibodies. Full slides were scanned using an Aperio ScanScope GL (Aperio Technologies, Vista, CA, USA) and analyzed for positive pixel area (Ly-6G) or positive pixel intensity (Caspase 3) using the Aperio Positive Pixel Analysis v9.1 algorithm.

Qualitative Real-Time Polymerase Chain Reaction

RNA was extracted from frozen livers using the TRIZOL method (Invitrogen, Carlsbad, CA, USA). DNAse treatment was performed using Turbo DNAse-free (Ambion, Austin, TX, USA). cDNA was synthesized using Superscript II RT and random hexamer primers (Invitrogen). A relative quantification PCR was performed to determine gene expression (Applied Biosystems, Foster City, CA). β-actin was used as housekeeping gene.

The primers used were as follows:

Heme Oxygenase-1 (HO-1)
Forward: CGAGGGAAACCCCAGATCA
Reverse: TTGCCAACAGGAAGCTGAGA

B-Cell Lymphoma-2 (Bcl-2)
Forward: CTGGGATGCCTTTGTGGAA
Reverse: TCAGAGACAGCCAGGAGAAATCA

Bcl-2 Associated X protein (BAX)
Forward: CAAGAAGCTGAGCGAGTGTCTC
Reverse: AATCATCCTCTGCAGCTCCATATT

Tumor Necrosis Factor alpha (TNFα)
Forward: ACAAGGCTGCCCCGACTAC
Reverse: TGACTTTCTCCTGGTATGAGATAGCA

Interleukin-6 (IL-6)
Forward: CGCTATGAAGTTCCTCTCTGCAA
Reverse: GTAGGGAAGGCCGTGGTTGT

alpha Smooth Muscle Actin (αSMA)
Forward: GAGAAAATGACCCAGATTATGTTTGA
Reverse: GGACAGCACAGCCTGAATAGC

Collagen 1a
Forward: GGAGAGTACTGGATCGACCCTAAC
Reverse: CTGACCTGTCTCCATGTTGCA

Hypoxia Inducible Factor-1 alpha (HIF-1α)
Forward: CTCAGAGGAAGCGAAAATGGA
Reverse: CAGTCACCTGGTTGCTGCAATA

PCR was performed in a total volume of 20 µl containing 10 ng cDNA template and 10 µl PCR-mastermix (Eurogentec). The Thermal Profile was 15 minutes at 95ºC, followed by 40 cycles of 15 seconds at 95ºC and 1 minute at 60 ºC. The average Ct-values for the target genes were subtracted from the average β-actin Ct-values to yield the delta Ct. Results were expressed as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data were analyzed using GraphPad PRISM 5.0 (GraphPad, San Diego, CA, USA) or SPSS 14.0 (SPSS Inc., Chicago, IL, USA) using, the Mann-Whitney U test or the Kruskall Wallis test where appropriate. Dunns postcorrection was applied where multiple comparisons were made. Normality was tested using the Kolmogorov–Smirno test. $p<0.05$ was considered statistically significant. All data are expressed as the mean ± SEM (Standard Error of the Mean) unless otherwise indicated.

RESULTS

Respirometry

Measurement of CO_2 production using the respirometry system indicated that mice enter a hypometabolic state within 5-10 minutes after the start of exposure to 100 ppm H_2S (Figure 1A). Our experiments indicate that CO_2 production is stable over longer periods of time when core body temperature is maintained. On the basis of these results, we chose a pretreatment period of 30 minutes for our ischemia experiments, to ensure that the mice would enter a stable state of hypometabolism (Figure 1B). CO_2 production of the animals just prior to the start of the ischemia procedure was measured in control and H_2S treated animals, verifying that mice had

entered a hypometabolic state in our IRI experiments. Relative CO_2 production was reduced by 38% ($p<0.001$) in H_2S treated animals (Figure 1C).

Necrosis

Necrotic hepatic surface area in the ischemic lobes was significantly increased after 24 hours of reperfusion in the IRI group ($p<0.05$), while H_2S treatment attenuated the onset of necrosis (Figure 2A). On average, after 6 hours of reperfusion, IRI animals had 7.4% necrotic surface area, while H_2S -treated animals had 13.2% (p=ns). After 24 hours, the IRI group had 46.2% necrotic area, while treatment with H_2S had only 7.5% necrotic area ($p<0.01$). Examples of the relative necrotic areas are shown in Figure 2B, in which necrosis was artificially colored red.

Serum AST, ALT and LDH

Serum levels of AST and ALT were both significantly higher in the IRI group at 1, 6 and 24 hours after reperfusion compared to sham-operated animals (Figure 3A,B). This increase was significantly lower in the IRI + H_2S group at 1 and 24 hours, but not at 6 hours after reperfusion. The reduction

Figure 1

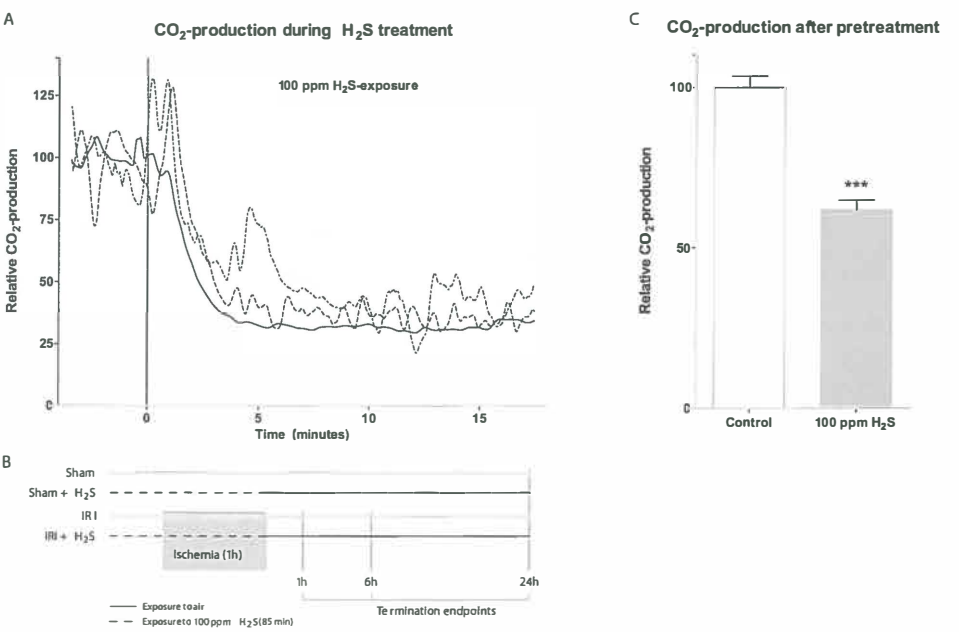


Figure 1 - H_2S induced hypometabolism and experimental design.
(A) CO_2-production of three separate, awake mice before and during treatment with 100 ppm H_2S. Exposure to H_2S rapidly reduces CO_2 production in these animals, indicating the induction of a hypometabolic state. (B) Experimental design, showing the H_2S treatment regimens used (dashed line indicates treatment with 100 ppm H_2S). (C) Average CO_2 production of anesthetized control and H_2S-treated mice at the end of the pretreatment period, just before ischemia. H_2S treated animals had significantly lower CO_2-production, indicating a successful induction of hypometabolism. (*** - $p<0.001$).

Figure 2

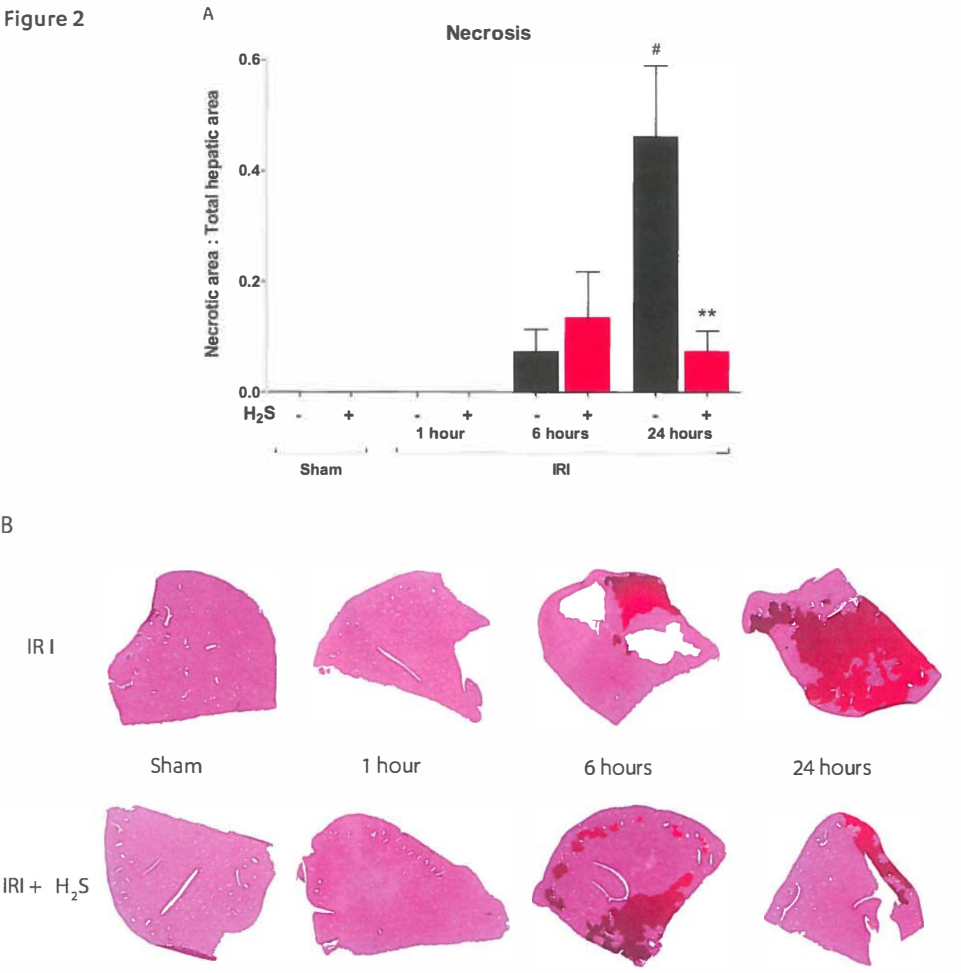


Figure 2 - H_2S reduces hepatic necrosis induced by ischemia.
(A) Relative necrotic area in haematoxylin-eosin (HE) stained hepatic sections, showing massive necrosis in the IRI group after 24 hours of reperfusion, which was significantly lower in the livers of H_2S treated animals. (B) Representative images of HE stained hepatic sections from all different treatment groups. Necrotic area was artificially colored red. (** - $p<0.01$ vs. IRI, # - $p<0.05$ vs. Sham).

in AST was 68.2% at 1h ($p<0.05$) and 75.4% after24h ($p<0.05$). The reduction in ALT was 50.9% after 1h ($p<0.05$) and 87.5% after 24 hours ($p<0.05$). LDH levels in the serum of IRI mice was significantly increased at 1 and 6 hours after reperfusion, which was significantly attenuated by H_2S treatment at 1 hour (25.1% lower, $p<0.05$), but not at 6 or 24 hours of reperfusion (Figure 3C).

Apoptosis

Immunohistochemistry for active Caspase 3 showed a substantial increase in apoptotic cells after 24 hours of reperfusion, especially in the peri-necrotic areas. This increase was fully

Figure 3

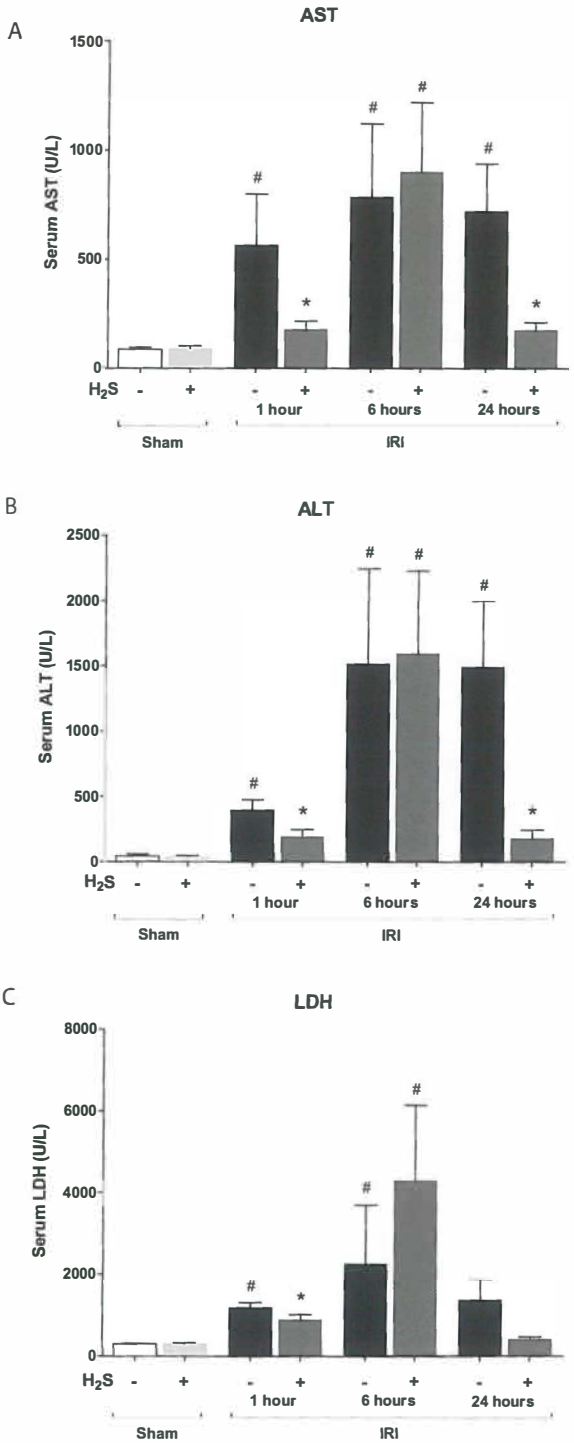


Figure 3 - Serum AST, ALT and LDH levels are attenuated by H_2S treatment.
(A,B) Serum AST and ALT levels show an increase at 1, 6 and 24 hours after ischemia, which was significantly reduced by pretreatment with H_2S at 1 and 24 hours of reperfusion. At 6 hours there was no significant difference between IRI + H_2S -treated animals and the IRI group. (C) Serum LDH levels were increased in IRI animals at 1 and 6 hours after reperfusion. H_2S significantly attenuated the increase at 1 hour of reperfusion, but not at 6 hours. (* - $p<0.05$ vs. IRI, # - $p<0.05$ vs. Sham).

prevented by H_2S treatment ($p<0.05$, Figure 4A). Livers from H_2S -treated animals had active Caspase 3 staining intensity comparable to sham-operated animals. Representative examples of active Caspase 3 staining are shown in Figure 4B-E. mRNA expression for the anti-apoptotic gene Bcl-2 were also significantly increased in the ischemic lobes of IRI animals after 24 hours (Figure 4F), while this increase was not seen in IRI + H_2S treated animals ($p<0.05$), indicating a sustained mitochondrial integrity. BAX gene expression was also significantly lower in H_2S treated animals (Figure 4G).

Inflammation

TNFα mRNA expression was significantly increased in the ischemic hepatic lobes of IRI animals 24 hours after reperfusion ($p<0.05$, Figure 5A). No significant increase of TNFα mRNA was seen in IRI + H_2S treated animals ($p<0.05$). Hepatic IL-6 mRNA levels were massively increased in IRI animals, while no increase was detected in IRI + H_2S treated animals ($p<0.05$, Figure 5B). Similarly, IL-1β mRNA levels were induced 24 hours after ischemia, and this increase was completely prevented by H_2S treatment (Supplementary table 1). IL-18 levels were not differentially expressed in any group (Supplementary table 1). Massive influx of Ly-6G positive granulocytes as assessed by immunohistochemistry was observed at 24 hours after reperfusion in IRI animals, while IRI + H_2S treated animals had greatly reduced increase in granulocytes in the ischemic hepatic lobes ($p<0.01$, Figure 5C). Figure 5D-G show representative sections of Ly-6G stained hepatic sections, demonstrating the extent of granulocyte influx into the necrotic areas in IRI animals.

Superoxide production

Superoxide levels in the hepatic sections were significantly increased in IRI animals at 6 and 24 hours after reperfusion (Figure 6, $p<0.01$). H_2S treatment significantly attenuated the increase in superoxide at 6 and 24 hours. ($p<0.01$).

HO-1, αSMA and Collagen-1a gene expression

The expression of heme oxygenase-1 (HO-1) mRNA was increased after 6 hours of reperfusion, in ischemic lobes as well as in non-ischemic lobes. H_2S did not significantly modulate this increase at the 6h time point. At 24 hours, mRNA expression was significantly increased in ischemic lobes of IRI animals ($p<0.05$), but not in non-ischemic lobes (Figure 7A,B). H_2S treatment significantly reduced the expression of HO-1 at the 24h time point in ischemic lobes, and relative to the non-ischemic lobe ($p<0.05$, Figure 7C).

Early expression of pro-fibrotic genes was assessed by investigating mRNA expression of αSMA and Collagen-1a (Supplementary table1). The expression of both genes in the ischemic lobes was reduced in IRI + H_2S treated animals compared to IRI after 24 hours of reperfusion $p<0.01$), although the changes in expression relative to the non-ischemic lobes were not significantly modulated.

HIF-1α gene expression

No significant differences in HIF-1α gene expression were detected in ischemic or non-ischemic lobes at all time points (Supplementary Table 1).

Figure 4

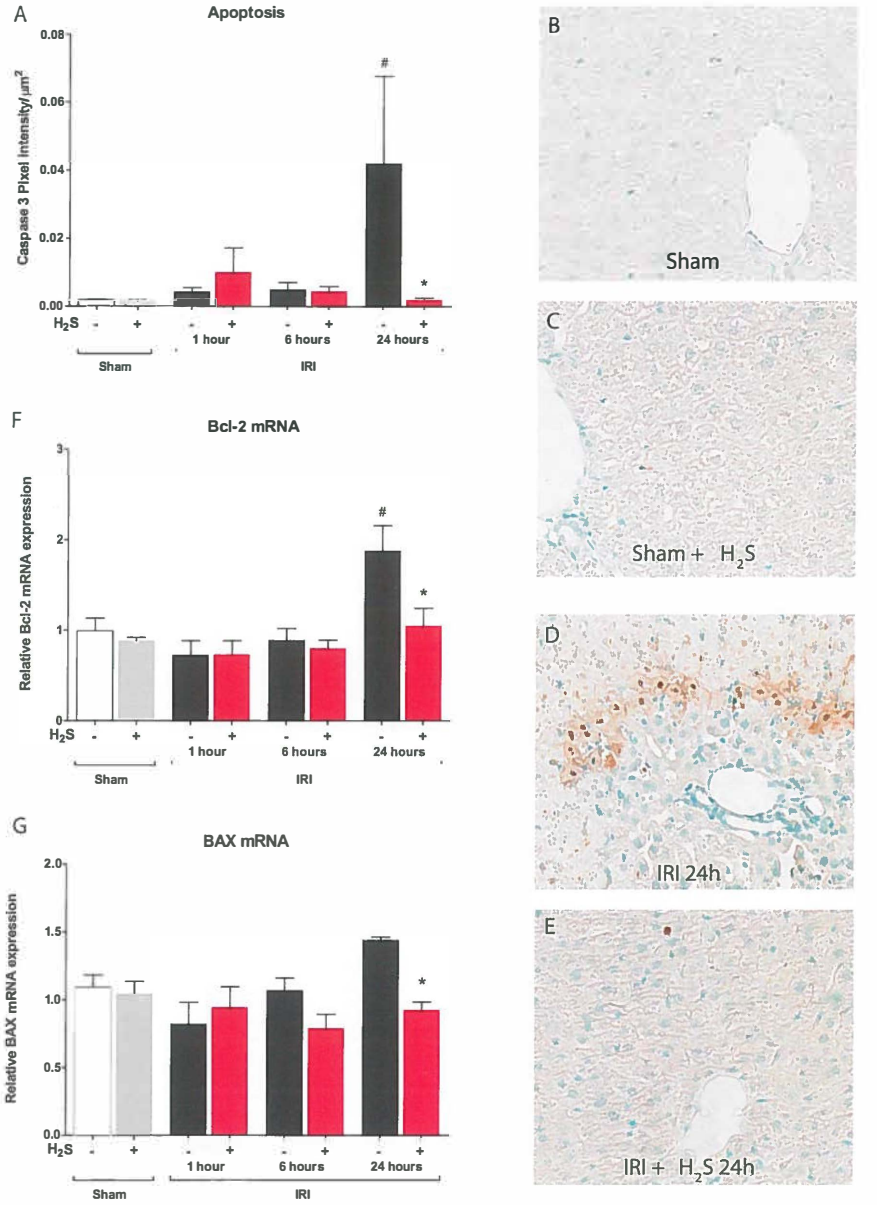


Figure 4 - H_2S treatment reduces apoptosis of hepatocytes.
(A) IRI induced massive apoptosis of hepatocytes, as measured by active Caspase 3 immunohistochemistry. H_2S treatment prevented the onset of apoptosis in the ischemic lobes. (B-E) Representative images of hepatic sections stained for active Caspase 3 using immunohistochemistry. mRNA levels of the anti-apoptotic gene Bcl-2 (F) and pro-apoptotic BAX (G) indicate the activation of the mitochondrial apoptosis pathway in livers exposed to IRI. mRNA levels were significantly lower in H_2S treated animals. (* $p<0.05$ vs. IRI, # - $p<0.05$ vs. Sham).

Figure 5

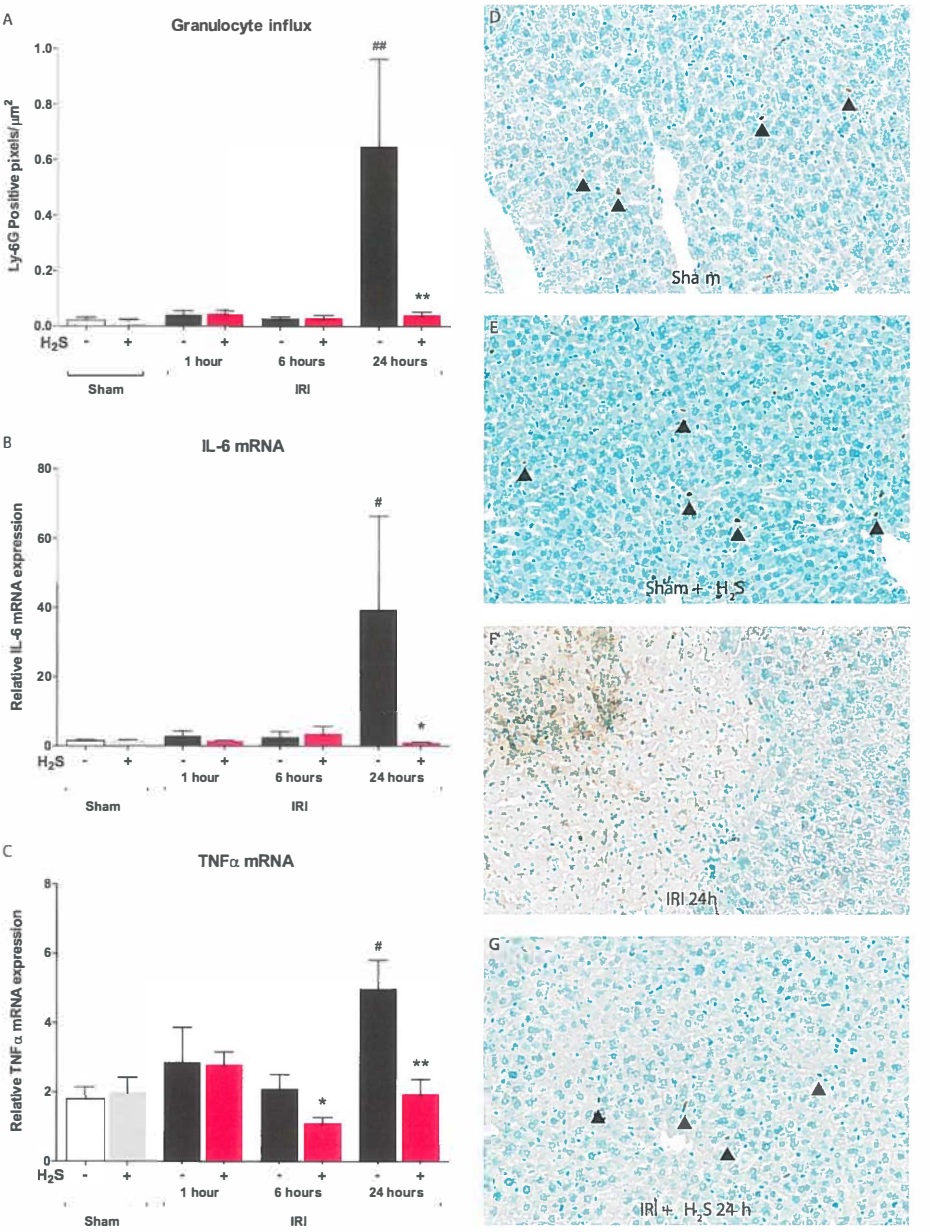


Figure 5 - H_2S treatment reduces inflammation and influx of granulocytes.
(A) Influx of Ly-6G positive granulocytes was assessed using immunohistochemistry. Influx of granulocytes was very high and was completely prevented by H_2S pretreatment. (B) Hepatic expression of TNFα mRNA is increased 24 hours after reperfusion and is significantly lower in H_2S treated animals at 6 and 24 hours of reperfusion. (C) IL-6 mRNA expression is massively higher after 24 hours of reperfusion and is not induced in H_2S treated animals. (D-G) Representative images of ►

DISCUSSION

Hepatic ischemia/reperfusion injury in mice can be prevented to a great extent by inducing a hypometabolic state with gaseous H_2S. In all of the dimensions of hypoxic injury that were investigated – including necrosis, apoptosis, inflammation and reactive oxygen species – H_2S treatment reduced deleterious effects, in some parameters to such an extent that damage was comparable to sham levels.

Several studies have reported on the use of H_2S in hypoxic injury, and the majority of studies show beneficial effects of H_2S treatment in models of shock[26], cardiac arrest[27] and in cardiac-[22,28], intestinal-[29], pulmonary-[30] and renal ischemia[31,32]. Two studies have shown beneficial effects of H_2S in hepatic IRI[33, 34]. These studies use soluble salts as donors of H_2S in solution (Na_2S or NaHS), and show that H_2S can reduce apoptosis and hepatic damage after IRI. They differ in the time of administration, however, one paper injecting before ischemia, and the other just before reperfusion. The unique perspective our study offers is the use of gaseous administration of H_2S and the induction of a hypometabolic state, linking the protective properties of H_2S to the reduction in the O_2-demand of the liver.

The increase in serum ALT, AST and LDH levels at 6 hours of reperfusion was equal in IRI and IRI + H_2S treated animals. This finding may well be related to the hepatic necrosis that was found at 6 hours of reperfusion, and suggests that H_2S cannot prevent all damage caused by hepatic IRI. Alternately, this could be a toxic effect of H_2S. Unfortunately, we did not include a group of sham-operated animals terminated 6 hours after the procedure, which would likely have shed more light on this observation. However, we believe it is unlikely that H_2S would induce such a massive increase in ALT, AST and LDH without showing any histological signs of necrosis, apoptosis or inflammation in sham operated animals.

The reduction in active Caspase 3 staining seen in H_2S treated animals indicated that H_2S can prevent the activation of apoptosis pathways. Since H_2S has been shown to protect mitochondrial integrity during hypoxia[22,31], we investigated if the mitochondrial apoptosis pathway would be activated in IRI animals. For this purpose, we measured the expression of Bcl-2 and found a significant increase in Bcl-2 mRNA after IRI. This increase was not found in livers from the IRI + H_2S group, indicating that the onset of mitochondrial degradation by IRI was prevented by H_2S treatment.

Treatment with H_2S prevented the production of TNFα and IL-6 mRNA after ischemia. This reduction in the production of cytokines may have contributed to the massively reduced influx of granulocytes to the necrotic areas in the liver. Treatment with H_2S lowered the influx of granulocytes after renal ischemia/reperfusion injury[31]. H_2S has also been implicated before in modulating leukocyte adhesion[35] through a mechanism involving the activation of K_{ATP} channels, although we did not test the role of K_{ATP} channels in this study. Nevertheless, there

► hepatic sections stained for granulocytes using Ly-6G antibody. Similar levels of granulocytes were seen in sham and H_2S treated animals, while the IRI group had very high influx of granulocytes, especially in the necrotic areas, as can be clearly seen in 5F. Arrowheads depict granulocytes in 5D-E,G (* - $p<0.05$, ** - $p<0.01$, both vs. IRI. # - $p<0.05$, ## - $p<0.01$, both vs. Sham).

Figure 6

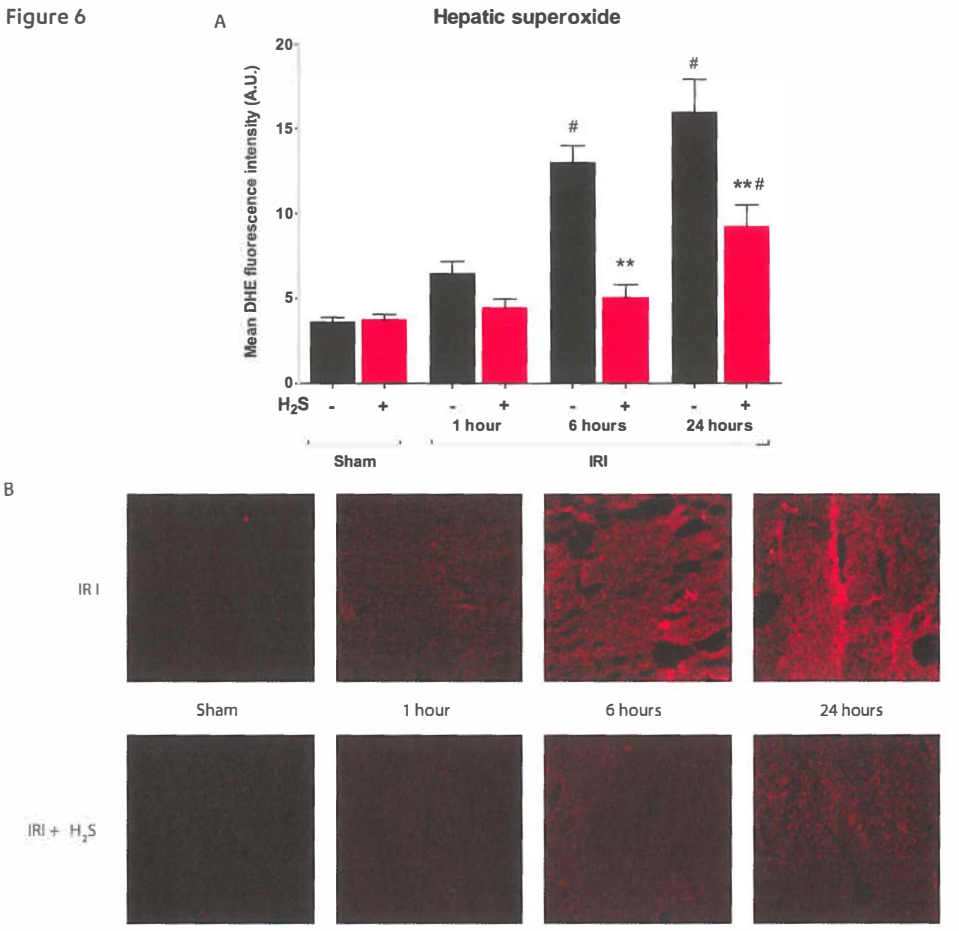


Figure 6 – Hepatic superoxide is reduced by H_2S treatment.
(A) Superoxide production in hepatic sections as assessed by DHE fluorescent imaging is significantly increased after IRI at 6 and 24 hours. Treatment with H_2S significantly reduced the extent of ROS at these timepoints. (B) Representative images of DHE fluorescence at all timepoints. (** $p<0.01$ vs. IRI, # - $p<0.05$ vs. Sham).

is no increase in granulocyte influx in H_2S treated animals after 24 hours of reperfusion, while there is an increase in necrotic area, indicating that although there is necrotic damage to these livers, there is reduced activation of signals that cause the influx of leukocytes.

The reduction in DHE fluorescence after IRI in H_2S treated animals indicates a reduced amount of ROS in the liver. H_2S can have direct scavenging effects on ROS, but can also have indirect effects, through increasing the amount of reduced glutathione (GSH). Whether direct or indirect ROS-scavenging mechanisms are at play here was not investigated. Another mechanism that could be involved is that reduction in oxidative metabolism and mitochondrial activity by H_2S inhibited the generation of ROS in the mitochondria.

Figure 7

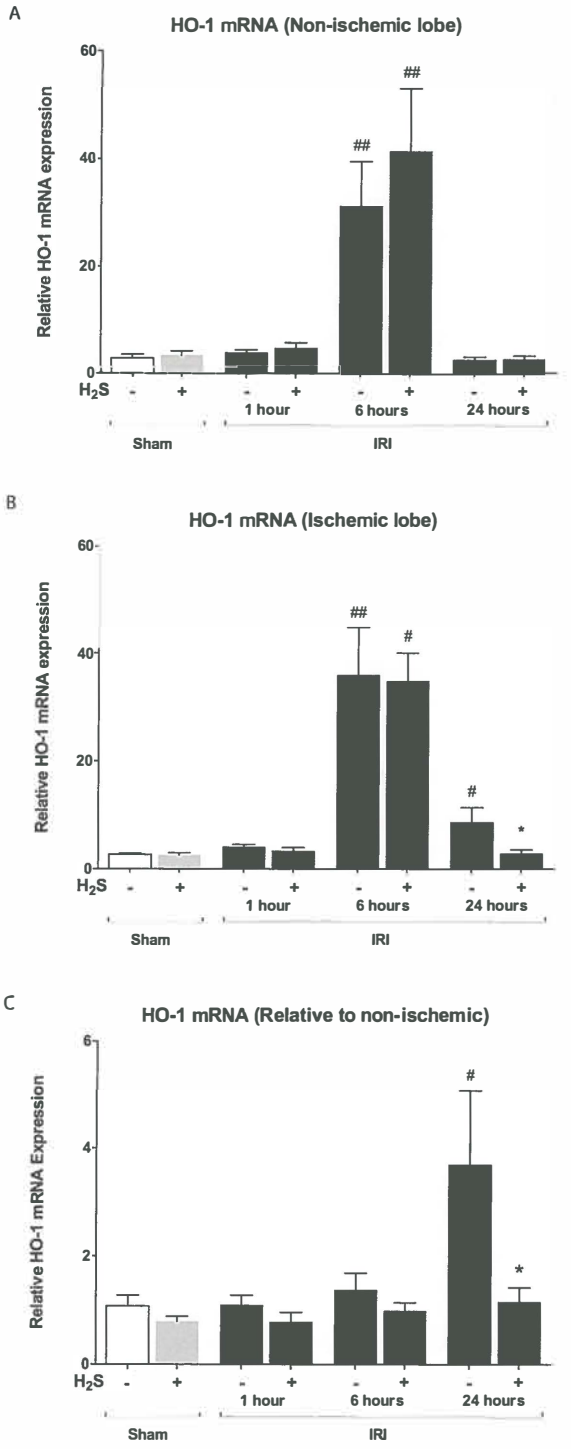


Figure 7 - HO-1 gene expression levels in ischemic and non-ischemic hepatic lobes.
(A, B) HO-1 mRNA expression is induced in non-ischemic and in ischemic lobes at 6 hours of reperfusion, indicating an effect not caused by ischemia. At 24 hours, expression of HO-1 is significantly higher in IRI compared to the IRI + H_2S group. (C) The ratio of ischemic to non-ischemic lobe expression of HO-1 indicates that induction of HO-1 is not involved in the protective mechanism of H_2S. (* - $p<0.05$ vs. IRI, # - $p<0.05$, ## - $p<0.01$, both vs. Sham).

Heme oxygenase-1 converts heme into biliverdin and CO, and is known as an enzyme that can be protective in models of oxidative stress[36]. It is known that gene expression of HO-1 is rapidly induced after hepatic ischemia/reperfusion injury[37]. Increased expression of HO-1 has been implicated as one of the mechanisms behind H_2S mediated protection in models of ischemia[28,38,39]. H_2S treatment did not induce the expression of HO-1 in sham-operated animals. We found a large increase in expression of HO-1 in ischemic as well as non-ischemic lobes 6 hours after the ischemia/reperfusion procedure, which was not modulated by H_2S treatment. This effect on HO-1 gene expression is most likely not caused by ischemia of the liver, but by the stress of the surgical procedure, anesthetics and/or analgesics, since expression in ischemic and non-ischemic lobes was similar. The significant increase in HO-1 expression in ischemic lobes after 24 hours of reperfusion, however, was attenuated by H_2S treatment, indicating that HO-1 expression is not implicated in the protective mechanism of H_2S in this model, but can be regarded as a marker of cellular or oxidative stress.

To investigate the main components of ischemia/reperfusion injury – necrosis, apoptosis, inflammation and fibrosis – we also investigated pre-fibrotic processes by looking at αSMA and Collagen-1a mRNA expression. There was significant modulation of the expression of both genes by H_2S in ischemic lobes, which indicated that treatment with H_2S might be able to prevent the onset of fibrotic mechanisms. However, when we corrected for the expression in the non-ischemic lobe there were no significant differences in expression. Also, the expression compared to sham-operated animals was not significantly changed, indicating that 24 hours of reperfusion is probably too early to assess ischemia induced fibrotic mechanisms after ischemia.

The mechanism behind the protective effects of hydrogen sulfide in hypoxic conditions might be related to its inhibitory effects on mitochondria. H_2S treatment can protect mitochondria from degradation, and improve viability in models of hypoxia and ischemia[22,31]. This could well be related to the inhibition of cytochrome c oxidase, or the scavenging of mitochondrial ROS. Fascinatingly, the induction of a hypometabolic state using H_2S can protect mice from an hypoxic atmosphere; where control mice died within 20 minutes after the onset of hypoxia, H_2S treated mice could survive for up to 6 hours[40]. This implies that the protection that we have seen during ischemia are possibly due to a reduced O_2 demand, thereby providing defense against hypoxia in the livers. In addition, it appears that H_2S can be used as an electron donor in mammalian cells[41]. When H_2S inhibits mitochondrial cytochrome c oxidase, cells might be able to use H_2S as an energetic substrate for anaerobic metabolism, allowing a low level of energy production during hypoxia. In addition, H_2S -induced vasodilatation might have a role in the protective effects of H_2S. Nevertheless, from the study presented in this paper it is impossible to say which combination of the many known effects of H_2S is responsible for the effects seen. We were unable to provide direct evidence towards the mechanisms that underlie H_2S mediated protection, only associative data.

The clinical potential of H_2S is diverse, and H_2S donors or H_2S-releasing compounds are being tested in different clinical settings now, including myocardial infarction, cardiopulmonary bypass and inflammatory conditions such as arthritis and inflammatory bowel disease. Ischemic conditions could greatly benefit from H_2S treatment. During surgical intervention H_2S might be valuable to

protect tissue from ischemia caused by temporary arterial clamping. However, some caution is required before these results can be translated to the human setting, since some of the effects of H_2S on mice could not be reproduced in larger animals, such as sheep[42]. However, others were able to induce lower core body temperature, O_2-consumption and CO_2-production in pigs[43].

In our view, one of the first feasible clinical applications of gaseous H_2S lies in the pretreatment of the brain dead (heart beating) organ donor. In addition to protecting the organ from ischemic damage, H_2S might also modulate the deleterious effects brain death has on organs that are to be transplanted[44]. However, the effects of brain death on H_2S-induced protection against IRI will need to be investigated. The fact that H_2S has protective effects in diverse organs makes H_2S a promising candidate to treat multi-organ donors prior to procurement. H_2S treatment will perhaps not only limit the extent of injury that occurs during the process of transplantation, but it might also increase the time livers can be preserved between explantation from the donor and implantation into the recipient. However, the potentially toxic effects of H_2S should be carefully monitored when used in a clinical setting.

In all, these data indicate that pretreatment with gaseous H_2S is a highly protective method to prevent ischemia/reperfusion injury of the liver, which makes it a promising candidate for use in a transplantation setting.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude towards Sanne Nieveld, Marian Bulthuis, Sippie Huitema, Petra J. Ottens, Janneke Wiersema-Buist, Pieter A. Klok, Jacco J. Zwaagstra, and Loes Butter for their excellent technical assistance.

This study was supported by grants from the Jan Kornelis de Cock-foundation, the Groningen University Institute for Drug Exploration (GUIDE) and Carburos Metálicos S.A.

The authors report no conflict of interest regarding this paper.

SUPPLEMENTARY TABLE

Supplementary Table 1 - mRNA expression of multiple genes in ischemic and non-ischemic lobes.

Gene	Treatment Group	Non-ischemic lobe			Ischemic lobe			Ischemic : Non ischemic ratio		
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
HO-1	Sham	2.94	1.62	ns	2.70	0.48	ns	1.07	0.44	ns
	Sham + H_2S	3.51	1.71		2.61	0.81		0.79	0.20	
	IRI - 1h	4.05	1.00	ns	4.16	0.86	ns	1.09	0.40	ns
	IRI + H_2S - 1h	4.89	2.12		3.37	1.39		0.78	0.38	
	IRI - 6h	31.21	18.41	ns	35.98	19.76	ns	1.37	0.69	ns
	IRI + H_2S - 6h	41.39	25.99		34.87	11.59		0.99	0.33	
	IRI 24h	2.67	1.09	ns	8.75	5.97	0.016	3.69	3.06	0.016
	IRI + H_2S - 24h	2.81	1.23		2.96	1.44		1.15	0.58	
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
TNFα	Sham	1.13	0.44	ns	1.99	1.18	ns	1.78	0.78	ns
	Sham + H_2S	0.87	0.66		1.67	1.41		1.97	1.01	
	IRI - 1h	1.17	0.23	ns	3.59	3.21	ns	2.84	2.22	ns
	IRI + H_2S - 1h	1.31	0.63		3.54	1.74		2.76	0.84	
	IRI - 6h	2.49	1.27	ns	4.32	1.79	ns	2.07	0.92	0.028
	IRI + H_2S - 6h	2.82	1.72		2.79	1.06		1.09	0.37	
	IRI - 24h	1.56	1.05	ns	7.00	3.27	0.004	4.94	1.91	0.004
	IRI + H_2S - 24h	0.94	0.96		1.12	0.80		1.91	0.96	
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
IL-6	Sham	0.22	0.08	ns	0.40	0.18	ns	1.56	0.43	ns
	Sham + H_2S	0.26	0.10		0.46	0.22		1.62	0.43	
	IRI - 1h	1.90	1.63	ns	2.63	1.17	ns	2.91	3.07	ns
	IRI + H_2S - 1h	2.35	1.03		2.38	0.64		1.29	0.82	
	IRI - 6h	0.46	0.24	ns	0.93	1.36	ns	2.48	3.40	ns
	IRI + H_2S - 6h	1.24	1.80		1.64	1.55		3.41	5.02	
	IRI - 24h	0.24	0.12	ns	6.08	8.33	0.048	37.00	62.03	0.016
	IRI + H_2S - 24h	0.40	0.16		0.33	0.16		0.84	0.61	

Supplementary Table 1 - Continued

Gene	Treatment Group	Non-ischemic lobe			Ischemic lobe			Ischemic : Non ischemic ratio		
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
IL-1β	Sham	0.37	0.33	ns	0.33	0.27	ns	0.94	0.26	ns
	Sham + H_2S	0.37	0.41		0.29	0.23		0.94	0.18	
	IRI - 1h	0.47	0.20	ns	0.69	0.49	ns	1.41	0.74	ns
	IRI + H_2S - 1h	0.50	0.16		0.74	0.11		1.57	0.28	
	IRI - 6h	0.26	0.15	ns	0.42	0.11	ns	1.97	0.85	ns
	IRI + H_2S - 6h	0.55	0.62		0.68	0.83		1.31	0.72	
	IRI - 24h	0.17	0.04	ns	1.22	1.07	0.02	7.19	6.73	0.004
	IRI + H_2S - 24h	0.24	0.10		0.24	0.09		1.05	0.20	
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
IL-18	Sham	1.01	0.43	ns	0.86	0.21	ns	0.91	0.18	ns
	Sham + H_2S	0.98	0.45		0.85	0.30		0.91	0.22	
	IRI - 1h	1.28	0.33	ns	0.91	0.18	ns	0.76	0.25	ns
	IRI + H_2S - 1h	1.25	0.17		0.93	0.13		0.76	0.20	
	IRI - 6h	1.40	0.21	ns	0.91	0.22	ns	0.65	0.15	ns
	IRI + H_2S - 6h	1.31	0.55		0.77	0.10		0.67	0.26	
	IRI - 24h	0.75	0.32	ns	0.58	0.13	ns	0.82	0.18	ns
	IRI + H_2S - 24h	0.80	0.36		0.69	0.36		0.91	0.30	
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
BAX	Sham	1.16	0.18	ns	1.24	0.15	ns	1.09	0.20	ns
	Sham + H_2S	1.08	0.27		1.10	0.23		1.04	0.19	
	IRI - 1h	1.19	0.36	ns	0.99	0.45	ns	0.82	0.35	ns
	IRI + H_2S - 1h	1.02	0.26		0.90	0.11		0.94	0.33	
	IRI - 6h	1.10	0.26	ns	1.14	0.19	ns	1.07	0.20	ns
	IRI + H_2S - 6h	1.38	0.16		1.12	0.49		0.79	0.23	
	IRI - 24h	1.15	0.18	ns	1.65	0.24	0.004	1.44	0.05	0.004
	IRI + H_2S - 24h	1.14	0.13		1.04	0.12		0.92	0.13	

Supplementary Table 1 - Continued

Gene	Treatment Group	Non-ischemic lobe			Ischemic lobe			Ischemic : Non ischemic ratio		
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
αSMA	Sham	0.42	0.33	ns	0.16	0.01	ns	0.50	0.22	ns
	Sham + H_2S	0.29	0.06		0.16	0.02		0.58	0.22	
	IRI - 1h	0.58	0.33	ns	0.34	0.13	ns	0.63	0.18	ns
	IRI + H_2S - 1h	0.49	0.09		0.40	0.13		0.88	0.32	
	IRI - 6h	0.51	0.27	ns	0.31	0.13	ns	0.72	0.48	ns
	IRI + H_2S - 6h	0.42	0.12		0.30	0.08		0.73	0.19	
	IRI - 24h	0.29	0.07	ns	0.29	0.09	0.008	1.04	0.31	ns
	IRI + H_2S - 24h	0.20	0.06		0.17	0.02		0.98	0.38	
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
Collagen 1a	Sham	1.78	2.01	ns	2.35	1.21	ns	2.82	2.80	ns
	Sham + H_2S	0.71	0.22		1.40	1.32		1.74	1.02	
	IRI - 1h	1.38	0.38	ns	1.24	0.46	ns	0.96	0.48	ns
	IRI + H_2S - 1h	1.49	0.31		2.03	0.87		1.37	0.62	
	IRI - 6h	2.41	2.49	ns	0.77	0.41	ns	0.55	0.44	ns
	IRI + H_2S - 6h	1.65	1.20		1.28	0.93		0.78	0.12	
	IRI - 24h	0.97	0.37	ns	3.73	0.52	0.006	4.41	2.11	ns
	IRI + H_2S - 24h	0.78	0.37		1.63	0.85		2.31	1.60	
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
HIF-1α	Sham	2.02	0.27	ns	2.01	0.09	ns	1.01	0.16	ns
	Sham + H_2S	2.06	0.40		1.84	0.33		0.91	0.24	
	IRI - 1h	1.55	0.51	ns	1.50	0.31	ns	1.04	0.27	ns
	IRI + H_2S - 1h	1.91	0.45		1.55	0.16		0.83	0.12	
	IRI - 6h	3.15	0.68	ns	2.96	0.79	ns	0.98	0.35	ns
	IRI + H_2S - 6h	3.61	0.62		3.59	1.02		0.99	0.19	
	IRI - 24h	1.56	0.37	ns	2.06	0.50	ns	1.38	0.50	ns
	IRI + H_2S - 24h	1.53	0.65		1.85	0.39		1.55	1.03	

REFERENCES

1. Fellström B, Aküyrek LM, Backman U, Larsson E, Melin J, Zezina L. Postischemic reperfusion injury and allograft arteriosclerosis. Transplant Proc. 1998;30(8):4278-80.
2. Howard TK, Klintmalm GB, Cofer JB, Husberg BS, Goldstein RM, Gonwa TA. The influence of preservation injury on rejection in the hepatic transplant recipient. Transplantation. 1990;49(1):103-7.
3. Kupiec-Weglinski JW, Busuttil RW. Ischemia and reperfusion injury in liver transplantation. Transplant Proc. 2005;37(4):1653-6.
4. Selzner M, Rüdiger HA, Sindram D, Madden J, Clavien PA. Mechanisms of ischemic injury are different in the steatotic and normal rat liver. Hepatology. 2000;32(6):1280-8.
5. Selzner N, Rudiger H, Graf R, Clavien P-A. Protective strategies against ischemic injury of the liver. Gastroenterology. 2003;125(3):917-36.
6. Reiffenstein RJ, Hulbert WC, Roth SH. Toxicology of hydrogen sulfide. Annu Rev Pharmacol Toxicol. 1992;32:109-34.
7. Wang R. The gasotransmitter role of hydrogen sulfide. Antioxid Redox Signal. 2003;5(4):493-501.
8. Fiorucci S, Antonelli E, Mencarelli A, Orlandi S, Renga B, Rizzo G, et al. The third gas: H_2S regulates perfusion pressure in both the isolated and perfused normal rat liver and in cirrhosis. Hepatology. 2005;42(3):539-48.
9. Wang R. Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? FASEB J. 2002;16(13):1792-8.
10. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, et al. H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science. 2008;322(5901):587-90.
11. Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J. 2001;20(21):6008-16.
12. Zagli G, Patacchini R, Trevisani M, Abbate R, Cinotti S, Gensini GF, et al. Hydrogen sulfide inhibits human platelet aggregation. Eur J Pharmacol. 2007;559(1):65-8.
13. Shintani T, Iwabuchi T, Soga T, Kato Y, Yamamoto T, Takano N, et al. Cystathionine beta-synthase as a carbon monoxide-sensitive regulator of bile excretion. Hepatology. 2009;49(1):141-50.
14. Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, et al. Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proc Natl Acad Sci USA. 2009;106(51):21972-7.
15. Abe K, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci. 1996;16(3):1066-71.
16. Kimura H. Hydrogen sulfide as a neuromodulator. Mol Neurobiol. 2002;26(1):13-9
17. Geng B, Chang L, Pan C, Qi Y, Zhao J, Pang Y, et al. Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol. Biochem Biophys Res Commun. 2004;318(3):756-63.
18. Whiteman M, Armstrong JS, Chu SH, Jia-Ling S, Wong B-S, Cheung NS, et al. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? J Neurochem. 2004;90(3):765-8.
19. Whiteman M, Cheung NS, Zhu YZ, Chu SH, Siau JL, Wong BS, et al. Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? Biochem Biophys Res Commun. 2004;326(4):794-8.
20. Szabó C. Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov. 2007;6(11):917-35.
21. Blackstone E, Morrison M, Roth M. H_2S induces a suspended animation-like state in mice. Science. 2005;308(5721):518.
22. Elrod JW, Calvert J, Morrison J, Doeller JE, Kraus DW, Tao L, et al. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci USA. 2007;104(39):15560-5.
23. Khan AA, Schuler MM, Prior MG, Yong S, Coppock RW, Florence LZ, et al. Effects of hydrogen sulfide exposure on lung mitochondrial respiratory chain enzymes in rats. Toxicol Appl Pharmacol. 1990;103(3):482-90.
24. Porte RJ, Ploeg RJ, Hansen B, van Bockel JH, Thorogood J, Persijn GG, et al. Long-term graft survival after liver transplantation in the UW era: late effects of cold ischemia and primary dysfunction. European Multicentre Study Group. Transpl Int. 1998;11 Suppl 1:S164-7.
25. Leemans JC, Stokman G, Claessen N, Rouschop K, al. e. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest. 2005.
26. Morrison ML, Blackwood JE, Lockett SL, Iwata A, Winn RK, Roth M. Surviving blood loss using

hydrogen sulfide. The Journal of trauma. 2008;65(1):183-8.

27. Minamishima S, Bougaki M, Sips PY, Yu JD, Minamishima YA, Elrod JW, et al. Hydrogen sulfide improves survival after cardiac arrest and cardiopulmonary resuscitation via a nitric oxide synthase 3-dependent mechanism in mice. Circulation. 2009;120(10):888-96.
28. Calvert J, Jha S, Gundewar S, Elrod JW, Ramachandran A, Pattillo CB, et al. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. Circ Res. 2009;105(4):365-74.
29. Liu H, Bai X-B, Shi S, Cao Y-X. Hydrogen sulfide protects from intestinal ischaemia-reperfusion injury in rats. J Pharm Pharmacol. 2009;61(2):207-12.
30. Fu Z, Liu X, Geng B, Fang L-P, Tang C-S. Hydrogen sulfide protects rat lung from ischemia-reperfusion injury. Life Sci. 2008;82(23-24):1196-202.
31. Bos EM, Leuvenink HGD, Snijder PM, Kloosterhuis NJ, Hillebrands J-L, Leemans JC, et al. Hydrogen sulfide-induced hypometabolism prevents renal ischemia/reperfusion injury. J Am Soc Nephrol. 2009;20(9):1901-5.
32. Tripatara P, Patel NSA, Collino M, Gallicchio M, Kieswich J, Castiglia S, et al. Generation of endogenous hydrogen sulfide by cystathionine gamma-lyase limits renal ischemia/reperfusion injury and dysfunction. Lab Invest. 2008;88(10):1038-48.
33. Jha S, Calvert J, Duranski MR, Ramachandran A, Lefer D. Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. Am J Physiol Heart Circ Physiol. 2008;295(2):H801-6.
34. Kang K, Zhao M, Jiang H, Tan G, Pan S, Sun X. Role of hydrogen sulfide in hepatic ischemia-reperfusion-induced injury in rats. Liver Transpl. 2009;15(10):1306-14.
35. Zanardo RCO, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J. 2006;20(12):2118-20.
36. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev. 2006;86(2):583-650.
37. Su H, van Dam GM, Buis CI, Visser DS, Hesselink JW, Schuurs TA, et al. Spatiotemporal expression of heme oxygenase-1 detected by in vivo bioluminescence after hepatic ischemia in HO-1/Luc mice. Liver Transpl. 2006;12(11):1634-9.
38. Oh G-S, Pae H-O, Lee B-S, Kim B-N, Kim J-M, Kim H-R, et al. Hydrogen sulfide inhibits nitric oxide production and nuclear factor-kappaB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radic Biol Med. 2006;41(1):106-19.
39. Qingyou Z, Du J-B, Weijin Z, Hui Y, Tang C-S, Chunyu Z. Impact of hydrogen sulfide on carbon monoxide/heme oxygenase pathway in the pathogenesis of hypoxic pulmonary hypertension. Biochem Biophys Res Commun. 2004;317(1):30-7.
40. Blackstone E, Roth M. Suspended Animation-Like State Protects Mice from Lethal Hypoxia. Shock. 2007;27(4):370-2.
41. Goubern M, Andriamihaja M, Nübel T, Blachier F, Bouillaud F. Sulfide, the first inorganic substrate for human cells. FASEB J. 2007;21(8):1699-706.
42. Haouzi P, Nonet V, Chenuel B, Chalon V, Sponne I, Ogier V, et al. H_2S induced hypometabolism in mice is missing in sedated sheep. Resp Phys Neurobiol. 2008;160(1)109-15.
43. Simon F, Giudici R, Duy CN, Schelzig H, Sükrü O, Gröger M, et al. Hemodynamic and metabolic effects of hydrogen sulfide during porcine ischemia/reperfusion injury. Shock. 2008;30(4)359–64.
44. Bos EM, Leuvenink HGD, van Goor H, Ploeg RJ. Kidney grafts from brain dead donors: Inferior quality or opportunity for improvement? Kidney Int. 2007;72(7):797-805.

4

GASEOUS HYDROGEN SULFIDE PROTECTS AGAINST MYOCARDIAL ISCHEMIA / REPERFUSION INJURY IN MICE PARTIALLY INDEPENDENT OF A HYPOMETABOLIC STATE

PM Snijder
RA de Boer
EM Bos
JC van den Born
WT Ruifrok
I Vreeswijk – Baudoin
MCRF van Dijk
JL Hillebrands
HGD Leuvenink
H van Goor

Published in Public Library of Science One

Reference: PLoS One, 2013 8(5):e63291
Digital object identifier (DOI): 10.1371/journal.pone.0063291

ABSTRACT

Ischemia-reperfusion injury (IRI) is a major cause of cardiac damage following various pathological processes. Gaseous hydrogen sulfide (H_2S) is protective during IRI by inducing a hypometabolic state in mice which is associated with anti-apoptotic, anti-inflammatory and antioxidant properties. We investigated whether gaseous H_2S administration is protective in cardiac IRI and whether non-hypometabolic concentrations of H_2S have similar protective properties.

Male C57BL/6 mice received a 0, 10, or 100 ppm H_2S-AIR mixture starting 30 minutes prior to ischemia until 5 min pre-reperfusion. IRI was inflicted by temporary ligation of the left coronary artery for 30 min. High-resolution respirometry equipment was used to assess CO_2-production and blood pressure was measured using internal transmitters. The effects of H_2S were assessed by histological and molecular analysis.

Treatment with 100 ppm H_2S decreased CO_2-production by 72%, blood pressure by 14% and heart rate by 25%, while treatment with 10 ppm had no effects. At day 1 of reperfusion 10 ppm H_2S showed no effect on necrosis, while treatment with 100 ppm H_2S reduced necrosis by 62% ($p<0.05$). Seven days post-reperfusion, both 10 ppm ($p<0.01$) and 100 ppm ($p<0.05$) H_2S showed a reduction in fibrosis compared to IRI animals. Both 10 ppm and 100 ppm H_2S reduced granulocyte-influx by 43% ($p<0.05$) and 60% ($p<0.001$), respectively. At 7 days post-reperfusion both 10 and 100 ppm H_2S reduced expression of fibronectin by 63% ($p<0.05$) and 67% ($p<0.01$) and ANP by 84% and 63% ($p<0.05$), respectively.

Gaseous administration of H_2S is protective when administered during an cardiac ischemic insult. Although hypometabolism is restricted to small animals, we now showed that low non-hypometabolic concentrations of H_2S also have protective properties in IRI. Since IRI is a frequent cause of myocardial damage during myocardial infarction and cardiac transplantation, H_2S treatment might lead to novel therapeutic modalities.

INTRODUCTION

Ischemia-reperfusion injury (IRI) is the most important cause of myocardial damage and subsequent heart failure. Although IRI is most frequently caused by acute myocardial infarction (MI) with (early or late) reperfusion, it can also be observed following surgical procedures such as cardiopulmonary bypass or cardiac transplantation.[1,2] Myocardial IRI causes acute tissue responses characterized by inflammation and upregulation of inflammatory mediators. This process ultimately leads to irreversible fibrotic damage.[3,4] Despite major therapeutic developments, cardiovascular disease remains the leading cause of death in the western world.[5]

Hydrogen sulfide (H_2S) has drawn considerable attention for its role in various (patho) physiological processes. It is, in addition to nitric oxide and carbon monoxide, acknowledged as the third gasotransmitter, sharing many functions with these gases.[6] H_2S is endogenously produced and exerts fine, modulatory control over cellular functions by influencing an array of intracellular signaling processes. H_2S-producing enzymes and H_2S-plasma levels are reduced in various diseases.[7-9] Exogenously administered H_2S can reversibly induce a hypometabolic state in mice, during which it rapidly reduces O_2-consumption, CO_2-production, core body temperature, heart rate and breathing frequency.[10,11]

The most probable mechanism for these properties is the reversible inhibition of mitochondrial O_2-consumption and ATP-production through non-permanent binding of sulfide to the terminal enzyme in the electron transport chain, cytochrome c oxidase (complex IV).[12] It was thought that the reduced demand for oxygen during hypometabolism might be one of the protective mechanisms during ischemia. However, H_2S is also considered protective during other processes critically involved in myocardial IRI such as oxidation, inflammation and apoptosis. These cytoprotective features of H_2S make it an attractive candidate for therapeutic reduction of the damaging effects of hypoxia.[13,14]

The influence of gaseous administration of H_2S and the effects of hypometabolic and non-hypometabolic concentrations on the outcome of myocardial IRI remains to be elucidated. Some studies have explored the beneficial effects of soluble H_2S donors such as NaHS and Na_2S in myocardial IRI and other models of cardiac damage.[15-18] The preference for gaseous administration above injection with H_2S donors lies within accurate management of the concentration. As opposed to injection with soluble H_2S donors, gaseous H_2S is less difficult to dose and has a short wash-out period, leaving its positive effects behind.[11] Moreover, gaseous administration has proven to induce a hypometabolic state, while this has not been shown for intra-peritoneal or intra-venous administration of soluble H_2S.[10,11] Although H_2S does not appear to have hypometabolic effects in ambiently cooled large mammals, thereby questioning its therapeutic applications in humans, the beneficial effects of non-hypometabolic concentrations of H_2S have not been studied.[19,20] Since minimizing myocardial IRI has broad clinical implications and may have beneficial effects on cardiac surgical outcomes[1], we therefore investigated whether gaseous H_2S-treatment attenuates myocardial IRI in mice and whether non-hypometabolic concentrations exhibit similar protective properties.

MATERIALS AND METHODS

Ethics Statement

Procedures were in agreement with institutional and legislator regulations and approved by the Committee on the Ethics of Animal Experiments of the University Medical Center Groningen. Utmost effort was utilized to prevent suffering and minimize the numbers of mice required for each experiment.

Animals

Male C57BL/6 mice (6-8 weeks, Harlan, Zeist, the Netherlands) were housed at our animal research facility under standard conditions with a 12h light:dark cycle with free access to water and chow.

Telemetry

Blood pressure was measured telemetrically (n=4) using transmitters (TA11PA-C10; Data Sciences International, St Paul, MN, USA). Devices were placed through a midline abdominal incision under anesthesia (2% Isoflurane) and mice were placed on a heating pad to maintain body temperature at 37°C. The catheter was placed in the aorta and the transmitter body in the abdominal cavity. Animals recovered 7 days before commencing measurements. Data were recorded as 10-second averages every minute using Dataquest ART data acquisition system (Data Sciences International). Animals were treated with room air or a H_2S / air mixture in our respirometry system during measurements. For comparison of blood pressure and heart rate, the average of 20 minutes baseline measurement and 20 minutes of 10 ppm and 100 ppm H_2S treatment was determined. A crossover design was used in which all animals received all treatments in randomized order.

Respirometry

Measurement of CO_2-production was performed as described before.[21] Compressed air and 500 ppm H_2S / N_2 (Air Products, Amsterdam, the Netherlands) were mixed in a 4:1 ratio and in a 49:1 ratio resulting in a 100 ppm H_2S / 17% O_2 mixture and a 10 ppm H_2S / 17% O_2 mixture, respectively. CO_2-production was corrected for body weight and normalized to mean control values. Animals (n=11) were treated in a crossover model in randomized order and all received room air, 10 ppm H_2S and 100 ppm H_2S on different days. Baseline CO_2 measurements with room air were performed for 30 minutes followed by treatment with either a 10 or 100 ppm H_2S / 17% O_2 mixture for 30 minutes. Recovery with room air was measured for 30 minutes.

Myocardial ischemia/reperfusion and H_2S treatment

Mice were intubated and mechanically ventilated (n=77) with an O_2/ N_2 mixture in a 4:1 ratio, an O_2 / 100 ppm H_2S / N_2 mixture in a 4:1 ratio or a O_2 / 10 ppm H_2S / N_2 mixture in a 49:1 ratio at a frequency of 180/min with a tidal volume of 250 µl using a rodent ventilator (Harvard Midivent model 849). Treatment regimens (Sham n=15; IRI n=20; 10 ppm n=21; 100 ppm n=21) were randomly assigned and started 30 minutes prior to ischemia until 5 minutes pre-reperfusion. Myocardial IRI was inflicted by temporary ligation of the left anterior descending coronary artery (6-0 prolene suture) for 30 min through an incision in the fourth intercostal space under

anesthesia (75 mg/kg ketamine, 1 mg/kg medetomidine). After removing the ligature the heart was inspected for restoration of blood flow and muscle and skin layers were sutured with 5.0 vicryl. Body temperature was monitored with a rectal probe and maintained at 37°C using heat pads. Sham operated animals underwent the same procedure, except the placement of the ligature. Post-operatively, all mice received a subcutaneous injection of 50 µg/kg buprenorphin (Schering-Plough) for analgesic purposes and were allowed to recover from surgery at 37°C in a ventilated incubator. After 1 and 7 days mice were anaesthetized with 2% isoflurane in O_2 for collection of blood and organs. Blood was collected in heparin containing tubes, centrifuged for 10 minutes at 1000 rcf and plasma was collected and stored at -80°C. The hearts were rapidly excised and mid-papillary slices were fixed in 4% paraformaldehyde, paraffin-embedded and sections were cut for immunohistochemical analysis. Apical parts of the heart were snap frozen in liquid nitrogen and stored at -80°C for molecular analysis.

Plasma analysis

Cardiac damage was assessed by measuring high sensitive (hs) Troponin-T in plasma samples using a standard electrochemiluminescence immunoassay (Roche) in the clinical chemical laboratory.

Histopathological scoring

At 1 day of reperfusion the extent of necrosis was determined in haematoxylin-eosin stained sections. At 7 days of reperfusion the extent of fibrosis was determined in Masson stained sections. Both were examined in a blinded fashion. Sections were scanned using an Aperio ScanScope GS (Aperio Technologies, Vista, CA, USA). Total cardiac area, necrotic cardiac area and fibrotic cardiac area were determined using Aperio Imagescope software, and the ratio of necrotic cardiac surface area and fibrotic surface area to total cardiac surface area were determined. Representative photomicrographs were artificially colored indicating the extent of damage.[11,21]

Immunohistochemistry for Ly-6G

For granulocytes, paraffin-embedded sections were stained for Ly-6G using rat-anti-mouse Ly6G/C-FITC IgG2b antibody (AbCam, Cambridge, MA, USA), followed by rabbit-anti-FITC and HRP-conjugated goat-anti-rabbit antibodies. Slides were scanned using an Aperio ScanScope GL (Aperio Technologies, Vista, CA, USA) and analyzed for positive pixel area (Ly-6G) using the Aperio Positive Pixel Analysis v9.1 algorithm.

Qualitative Real-Time Polymerase Chain Reaction

RNA extraction, DNAase treatment[21] and cDNA synthesis[22] were performed as described. A relative quantification PCR was performed to determine gene expression (Applied Biosystems, Foster City, CA). β-actin and GAPDH were used as housekeeping genes.

The primers used were:

Fibronectin (NM_010233.1)
Forward: AGGAAATGTACTGAATGCTAGTACCCA
Reverse: TCAGATGGCAAAAGAAAGCAGA

ANP (NM_008725.2)
Forward: ACCCTCCTGGAGCTGCG
Reverse: ACCCCACTAGACCACTCATCTACAT

NOX2 (NM_007807.4)
Forward: GATGCAATAAGACTAGGCACAAACC
Reverse: CCATCTCATAACCAGAATAACTCAGGATA

NOX4 (NM_015760.4)
Forward: TGCACCAAACACAGAAGCACA
Reverse: AGCAGGGTATCACTCCATGAATTC

PCR was performed in a volume of 20 µl containing 10 ng cDNA and 15 µl PCR mastermix (SYBR GREEN Applied Biosystems; 5 ml P/N 4309155). The Thermal Profile was performed as described.[22] The average Ct-values for fibronectin, ANP, NOX2 and NOX4 were subtracted from the average β-actin Ct-values and the average of β-actin and GAPDH Ct-values to yield the delta Ct. Results were expressed as $2^{-\Delta Ct}$.

Cell culture

The H9c2 cell line (ATCC) is an immortalized line with characteristics of rat heart myoblasts. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Germany) containing 4.5 g/l glucose, 10% fetal calf serum (FCS; Bodinco, Alkmaar, the Netherlands), L-glutamine and penicillin (100 U/ml) streptomycin (100 µg/ml) (Lonza, Germany). Cells were cultured using 75 cm^2 collagen coated flasks (Corning, Schiphol-Rijk, Netherlands) in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C.

In vitro model of oxidative stress

H9c2 cells grown to 80-90% confluency were harvested using 3 ml trypsin EDTA (200 mg/l) after washing twice with Hank's Buffered Salt Solution (HBSS) (Lonza; Germany). Cells were cultured in a 24-well plate at a density of ~10.000 cells/well in 0.5 ml medium. After 24 hours cells were loaded with 15 µM Dihydroethidine (DHE). Culture plates were placed in a humidified chamber with 5% CO_2 on an automated inverted fluorescent microscope system (TissueFAXS system, TissueGnostics GMBH, Vienna, Austria) which makes sequential photomicrographs of 9 area's in each well every 5 minutes. After baseline measurements, cells were exposed to Antimycin (50 µg/mL) and NaHS (donor of H_2S in solution) in a concentration of 1 mM. Fluorescence intensity of every cell was analyzed using the TissueQuest software (TissueGnostics).

Statistical analysis

Data were analyzed using GraphPad PRISM 5.0 (GraphPad, San Diego, CA, USA) using two-way ANOVA, Mann-Whitney U, Friedman or Kruskall Wallis tests where appropriate. Bonferroni or Dunns post-hoc analysis was applied where multiple comparisons were made. Normality was tested using the Kolmogorov–Smirnov test. $p<0.05$ was considered statistically significant. All data are expressed as mean ± SEM (Standard Error of the Mean) unless otherwise indicated.

RESULTS

Effect of H_2S on CO_2 production, blood pressure and heart rate

Within 15 minutes of treatment with 100 ppm H_2S induced a state of hypometabolism, concomitant with a reduction in CO_2-production by an average of 72% compared to basal levels ($p<0.001$). Cessation of H_2S resulted in a rapid recovery of CO_2-production, where CO_2 concentrations raised to basal levels within 30 minutes ($p<0.001$). 100 ppm H_2S lowered blood pressure by 14% (103 *vs.* 120 mmHg, $p<0.05$) and heart rate by 25% compared to baseline (502 *vs.* 670 beats per minute, $p<0.05$) 10 ppm H_2S had no effect on CO_2 production, blood pressure and heart rate (Figure 1).

Figure 1

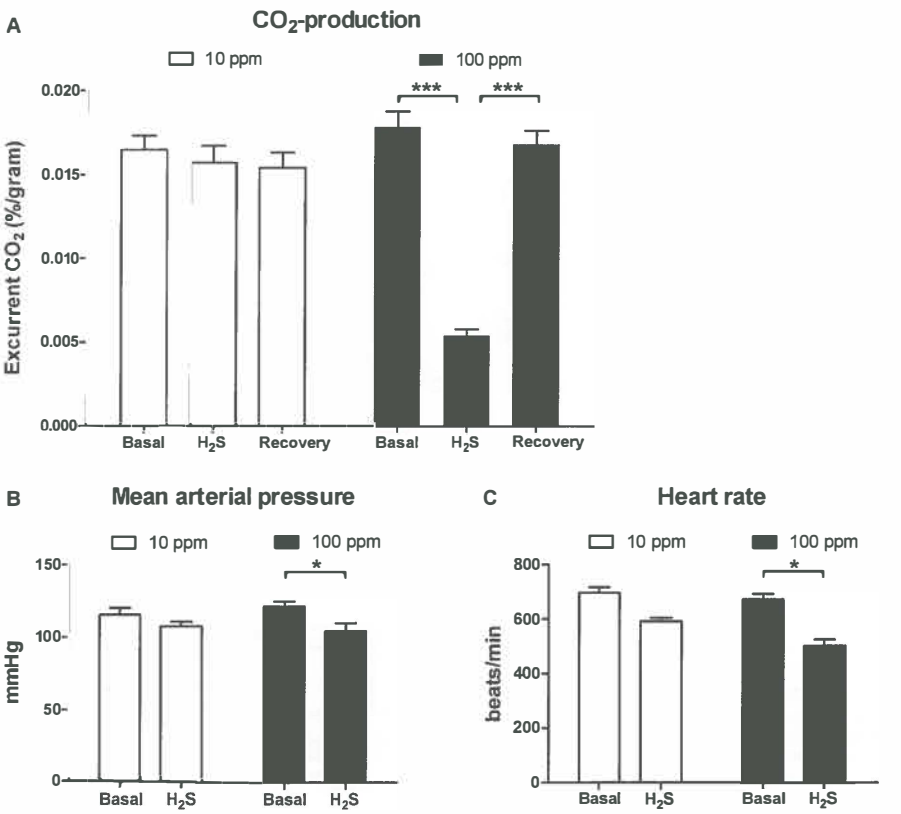


Figure 1 – High concentrations of H_2S induce a state of hypometabolism.
Within 15 minutes (A) CO_2-production decreased by 72% (***$p<0.001$) in mice (n=11) subjected to 100 ppm H_2S. Exposure to 10 ppm H_2S (n=11) did not induce a reduction in CO_2 production in these animals. Cessation of gaseous H_2S resulted in rapid recovery, within 30 minutes CO_2 levels returned back to baseline concentrations (***$p<0.001$). Administration of 100 ppm H_2S (n=4) resulted in a 14% decrease in (B) mean arterial pressure and a 25% decrease in (C) heart rate (*$p<0.05$). However exposure to 10 ppm H_2S (n=4) had no effect on mean arterial pressure or heart rate.

H_2S reduces myocardial damage

At 1 day of reperfusion cardiac IRI induced significant necrosis (Figure 2A) in animals exposed to 0 ppm H_2S when compared to sham animals ($p<0.001$) as indicated by infarct size. 10 ppm H_2S did not reduce the size of the necrotic area, while 100 ppm H_2S reduced infarct size by 62% ($p<0.05$) (Figure 2B). In mice treated with 10 ppm H_2S hs Troponin-T levels were not reduced 1 day post-reperfusion, while 100 ppm H_2S reduced hs Troponin-T levels by 47% ($p<0.05$) compared to IRI animals (Figure 2C). Fibrosis, as measured by collagen deposition in Masson stained sections (Figure 3A) at 7 days of reperfusion, was markedly increased in animals treated with 0 ppm H_2S when compared to sham-operated animals ($p<0.001$). Treatment with either 10 or 100 ppm of H_2S reduced collagen deposition to comparable levels (10 ppm: 59%, $p<0.01$; 100 ppm: 57%, $p<0.05$) (Figure 3B). Cardiac mRNA levels of fibronectin, a marker of myocardial fibrosis, were massively increased in 0 ppm H_2S treated animals ($p<0.01$), while no increase was detected in animals of both H_2S treated groups (10 ppm H_2S $p<0.05$; 100 ppm H_2S $p<0.01$) (Figure 3C). Seven days post-reperfusion hs Troponin-T levels were reduced by 59% ($p<0.05$) and 75% ($p<0.01$) in 10 ppm and 100 ppm H_2S treated mice, respectively (Figure 3D).

Figure 2

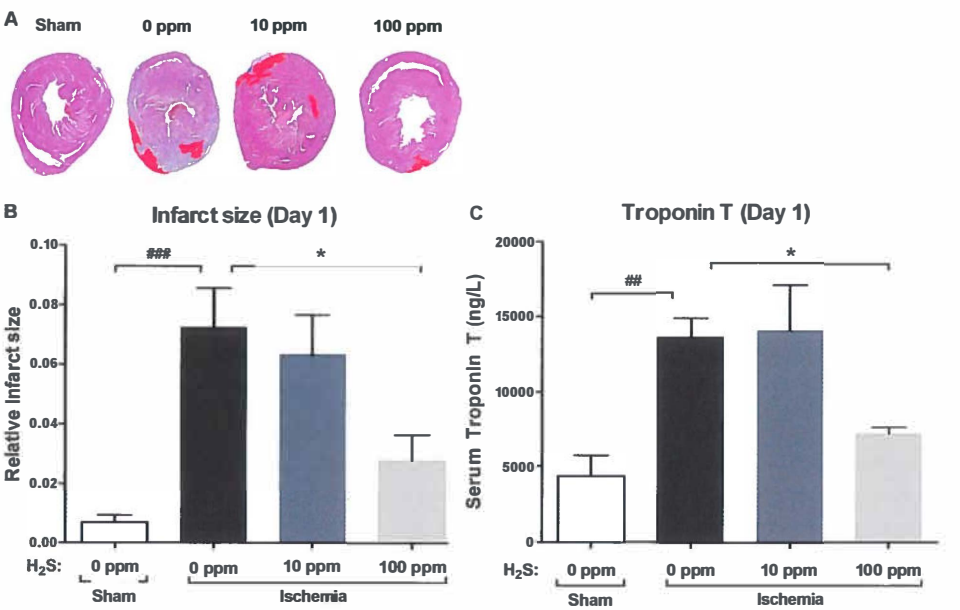


Figure 2 – Cardiac damage is reduced by 100 ppm H_2S at 1 day of reperfusion.
(A) Representative photomicrographs of haematoxylin-eosin stained cardiac sections with necrotic area artificially colored red, indicating the extent of necrotic damage found in each group at 1 day of reperfusion. (B) Cardiac IR induced a significant amount of necrosis in IRI animals exposed to 0 ppm H_2S (### $p<0.001$ vs. sham). In animals treated with 100 ppm H_2S necrosis was reduced by 62% (* $p<0.05$ vs. IRI) where as 10 ppm H_2S had no effect on necrosis. (C) At 1 day of reperfusion hs Troponin-T levels were elevated in IRI animals exposed to 0 ppm H_2S (## $p<0.01$ vs. sham). In the 100 ppm H_2S treated group hs Troponin-T levels were reduced compared to 0 ppm treated animals (* $p<0.05$), 10 ppm H_2S had no effect.

ANP-gene expression

mRNA expression of atrial natriuretic peptide (ANP), a marker for induction of the fetal gene program, was significantly increased in hearts of mice treated with 0 ppm H_2S compared to sham-operated animals at 7 days of reperfusion. In hearts of 10 and 100 ppm H_2S treated mice the relative ANP expression was significantly reduced compared to mice treated with 0 ppm H_2S ($p<0.05$) (Figure 3E).

Inflammation

One day post-reperfusion, Ly-6G-positive granulocytes were increased 12-fold in animals treated with 0 ppm H_2S compared to sham-operated animals ($p<0.001$). Exposure to 10 ppm and 100 ppm H_2S reduced granulocytes by 43% ($p<0.05$) and 60% ($p<0.001$), respectively (Figure 4).

NOX2 and NOX4 gene expression

To investigate ROS-related genes in vivo, we measured mRNA expression of nicotinamide adenine dinucleotide phosphate oxidase 2 and 4 (NOX2 and NOX4). At 1 day of reperfusion no significant differences were found in NOX2 and NOX4 mRNA expression. Seven days post-reperfusion, NOX2 and NOX4 expression were significantly increased in hearts of mice treated with 0 ppm H_2S compared to sham operated animals (NOX2: $p<0.05$, NOX4: $p<0.01$). NOX2 and NOX4 expression were not amplified in hearts of mice treated with 10 and 100 ppm H_2S compared to mice treated with 0 ppm H_2S ($p<0.05$) (Figure 5A and B).

Effect of H_2S on ROS production in vitro

Antimycin A induced ROS production in cultured H9c2 rat cardiomyoblasts was significantly reduced by treatment with NaHS. Live cell imaging of DHE fluorescence showed a massive increase in cytoplasmatic ROS production during treatment with Antimycin, whereas addition of NaHS to the medium markedly reduced this fluorescence signal ($p<0.001$) (Figure 6).

DISCUSSION

The major finding of this study is that administration of hypometabolic concentrations of gaseous H_2S during myocardial IR limits the extent of myocardial damage. Furthermore, non-hypometabolic concentrations of H_2S do not seem protective in the early phase after myocardial infarction, but attenuate ischemia associated processes such as fibrosis and ROS formation. Gaseous administration of H_2S appears to be an effective way to attenuate the outcome of myocardial IRI, with multiple mechanisms seemingly underlying the protective properties.

H_2S is cytoprotective during hypoxia in multiple organs. Beneficial effects of H_2S treatment have been reported in models of shock[23] and intestinal-[24], pulmonary-[25], hepatic-[21,26] and renal ischemia.[11] The cardio-protective effects of H_2S have been demonstrated in models of myocardial injury. However, most of these studies use injection with soluble H_2S donor compounds such as sodium hydrosulfide (NaHS) or sodium sulfide (Na_2S), while no results have been published on gaseous H_2S.[15-18,27] Gaseous administration might be applicable in patients who are being ventilated because of cardiogenic shock, a state of brain death or during transplantation procedures. Inhaled H_2S has beneficial effects in endotoxin induced systemic inflammation[28] and in experimental

Figure 3

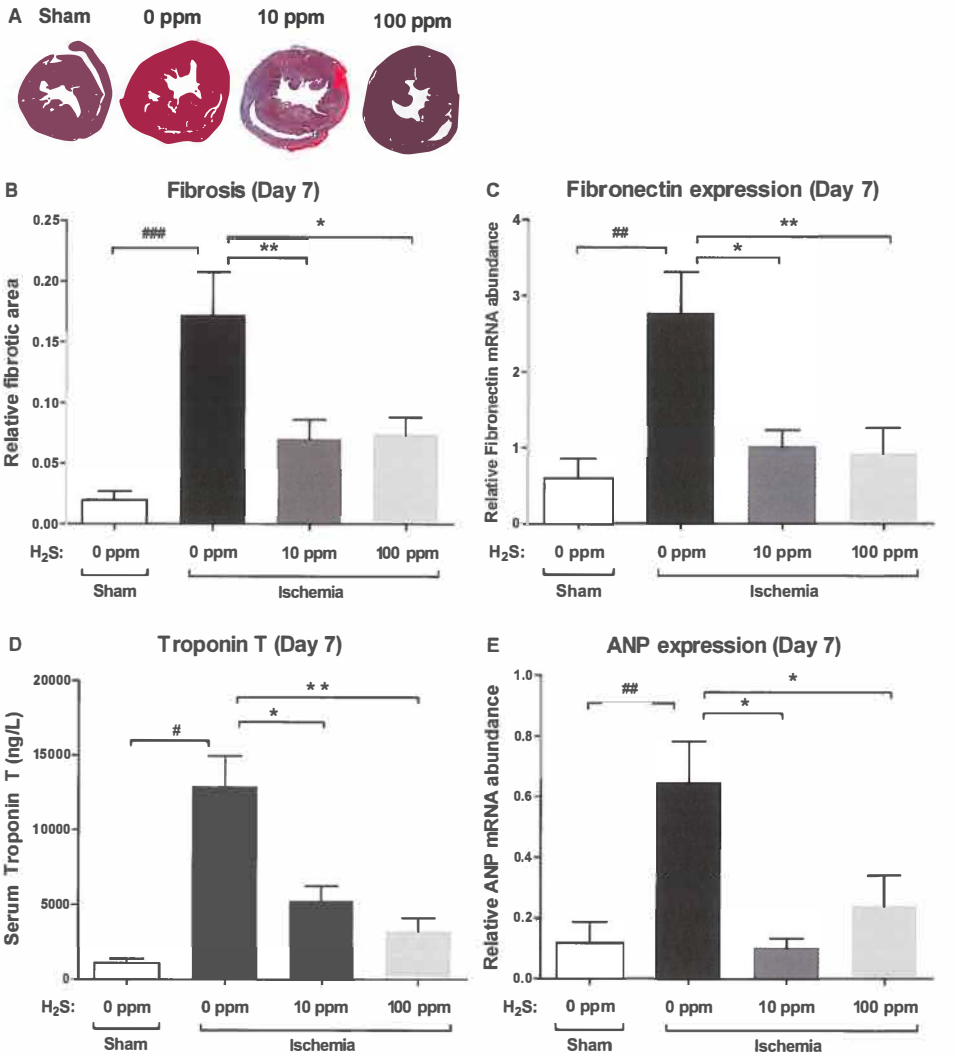


Figure 3 – Cardiac damage is reduced by 10 and 100 ppm H_2S at 7 days of reperfusion.
(A) Representative photomicrographs of Masson stained cardiac sections with fibrotic area artificially colored red, indicating the extent of fibrotic damage found in each group at 7 days of reperfusion. (B) Cardiac IR induced a significant amount of fibrosis in IRI animals exposed to 0 ppm H_2S (### p<0.001 vs. Sham). In animals treated with 10 ppm and 100 ppm H_2S fibrosis was significantly reduced (** p<0.01, * p<0.05 vs. IRI). (C) Expression of fibronectin at 7 days of reperfusion was increased in IRI animals (## p<0.01 vs. sham). Treatment with 10 and 100 ppm H_2S reduced the expression of fibronectin (* p<0.05, ** p<0.01 vs. IRI). (D) Seven days post-reperfusion hs Troponin-T levels were elevated in IRI animals exposed to 0 ppm H_2S (# p<0.01 vs. sham). Both 10 and 100 ppm H_2S reduced hs Troponin-T levels by 59% and 75%, respectively, as compared to animals treated with 0 ppm (* p<0.05, ** p<0.01 vs. IRI). (E) Expression of ANP mRNA at 7 days of reperfusion was increased in IRI animals (## p<0.01 vs. Sham). Treatment with 10 and 100 ppm H_2S reduced the expression of ANP (* p<0.05 vs. IRI).

Figure 4

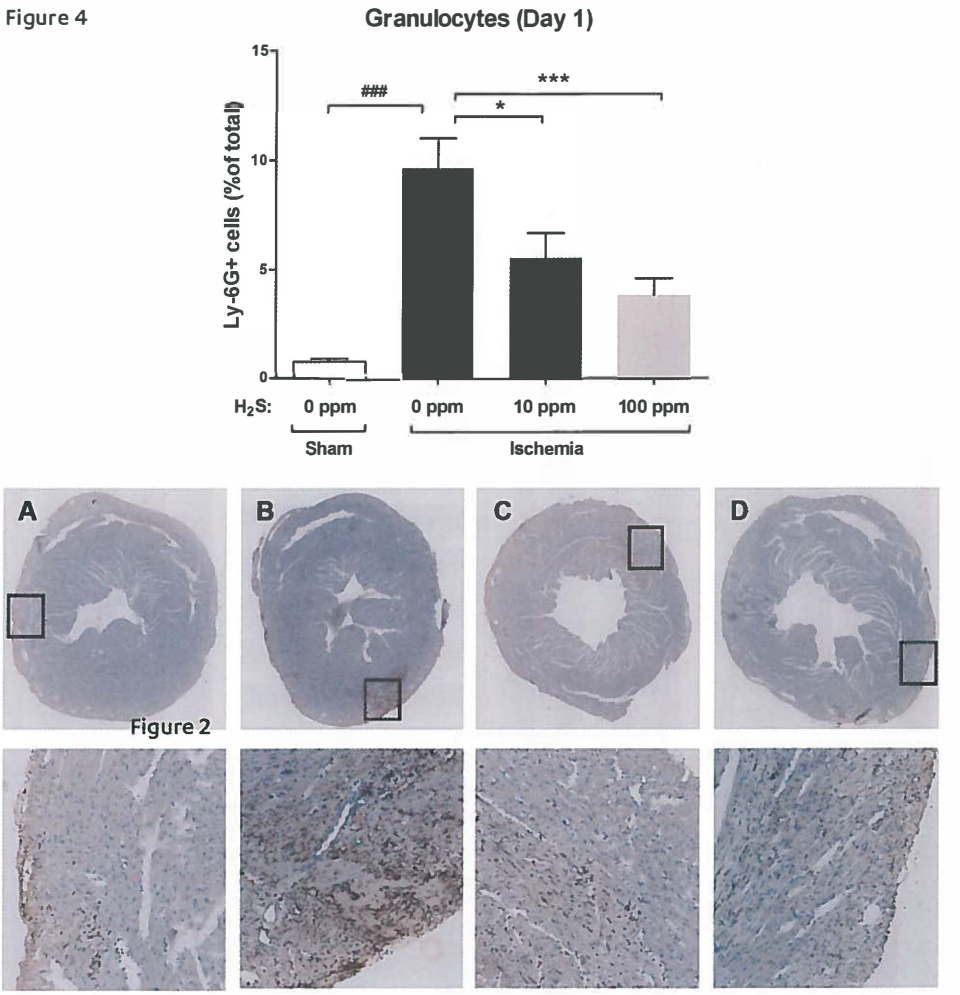


Figure 4 – H_2S reduces cardiac IRI induced inflammation.
There was a marked increase in granulocyte influx after cardiac ischemia ($p<0.001$) compared to sham animals. Exposure to 10 ppm and 100 ppm H_2S significantly reduced the influx of granulocytes in the infarct area (10 ppm $p<0.05$; 100pm $p<0.001$). Below are representative images from these stainings: (A) Sham (B) IRI, 0 ppm H_2S (C) IRI, 10 ppm H_2S (D) IRI, 100 ppm H_2S.

Parkinson's disease.[29] We previously showed protective effects of gaseous H_2S during renal and hepatic IRI.[11,21] The benefits of gaseous administration compared to injections with soluble H_2S donors lay within the management of the concentration. As opposed to injection with H_2S donors it is possible to administer the gas continuously with a stable dose over longer periods of time. Furthermore, when treatment is stopped the effects vanish rapidly while leaving its positive therapeutic effects behind. Also, gaseous administration at higher concentrations has proven to induce a hypometabolic, hibernation-like state in small animals like rodents.[10,11]

Figure 5

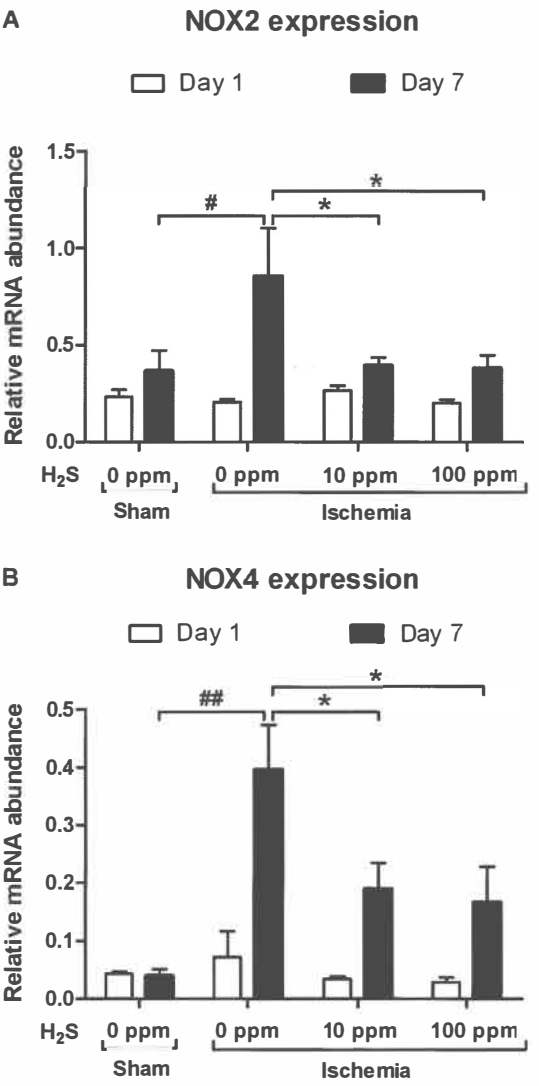


Figure 5 – H_2S attenuates NOX2 and NOX4 upregulation.
Expression of (A) NOX2 and (B) NOX4 mRNA was increased in IRI animals at 7 days post-reperfusion (NOX2: # $p<0.05$, NOX4: ## $p<0.01$ vs. Sham). Treatment with 10 and 100 ppm H_2S reduced the expression of both genes (* $p<0.05$ vs. IRI). After 1 day of reperfusion no differences were observed between all groups.

As previously shown, 100 ppm H_2S induces a hypometabolic state and lowers blood pressure, heart rate and CO_2-production, whereas 10 ppm H_2S does not.[10,11,30] A suspended animation-like state induced by H_2S protects mice from lethal hypoxia for periods up to 6 hours[31], suggesting that the induction of regulated, reversible and well-controlled hypometabolism in organs holds clinical promise in ischemia-reperfusion related damage where oxygen demand exceeds oxygen availability. Although there was no difference in cardiac necrosis between the 10 ppm treated group and 0 ppm treated group, treatment with 100 ppm significantly reduced necrosis at 1 day of reperfusion. High-sensitive Troponin-T (hsTnT) levels in serum were in line

Figure 6

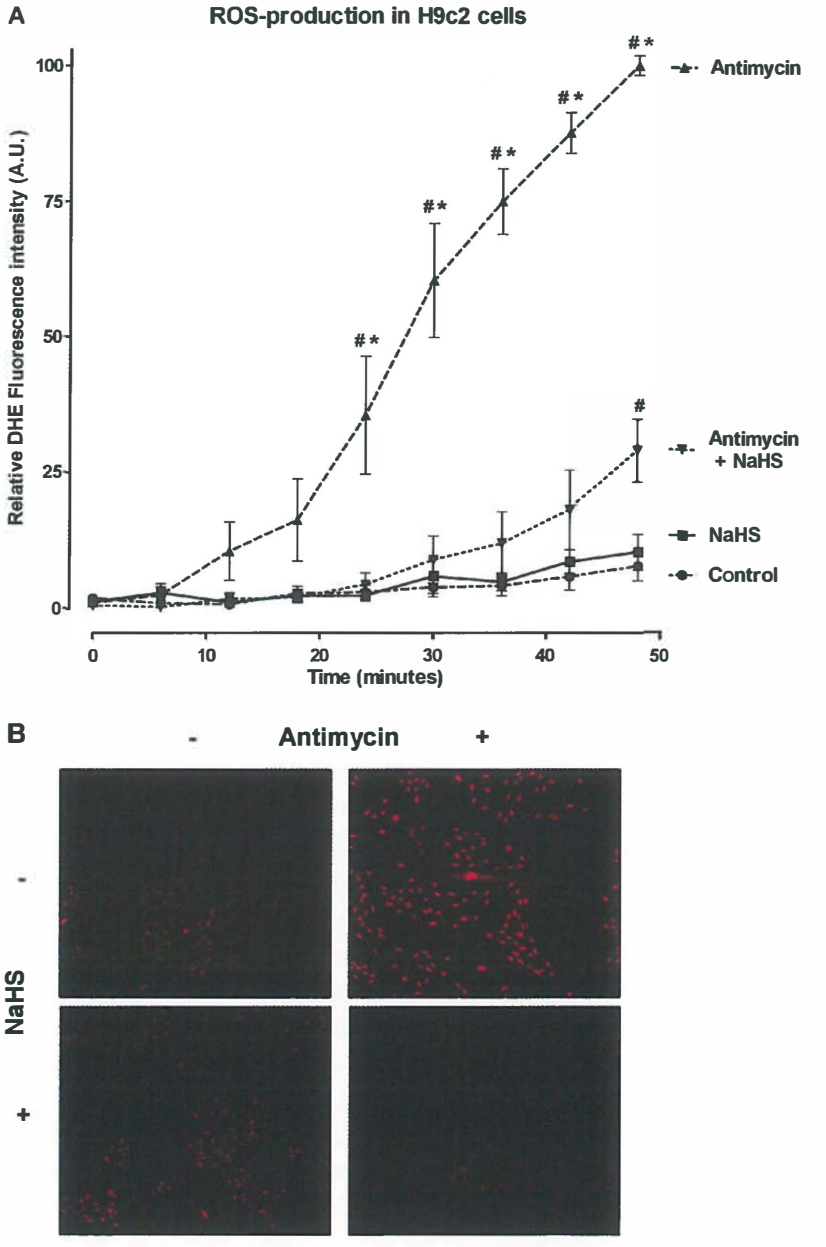


Figure 6 – H_2S reduces ROS production in cultured cardiomyoblasts.
(A) Antimycin (50 µg/ml) significantly induced ROS production in H9c2 cells from 24 minutes onwards compared to untreated control cells. Addition of 1 mM NaHS prevented the increase in ROS production. (# $p < 0.001$ vs. control; * $p<0.001$ vs. NaHS) (B) Representative photomicrographs of DHE stained H9c2 cells treated with antimycin and NaHS showing less DHE staining (red) in the NaHS treated cells as compared to cells treated with only antimycin.

with this finding. This suggests that H_2S in non-hypometabolic concentrations is not effective in preventing short-term necrosis caused by ischemia, and that the additional value of H_2S-induced hypometabolism lies in the early phase of IRI. Although, the protective effects of 100 ppm H_2S on necrosis might also be caused by a larger amount of H_2S leading to increased anti-oxidant effects, it is difficult to distinguish between the effects of hypometabolism and other effects attributed to a higher dose. However, inducing a suspended animation-like state might be restricted to small animals like rodents. The applicability of hypometabolism in larger animals is still under debate and we are far from developing therapeutic applications in reducing metabolic rate in the clinical setting with the use of H_2S.[19,20,32,33]

There are a number of potential mechanisms through which H_2S may exert its cardioprotective effects. Both 10 and 100 ppm of H_2S were proven anti-inflammatory as evidenced by reduced granulocyte influx into necrotic areas. Treatment with H_2S also lowered the influx of granulocytes after renal IRI.[11] Furthermore, H_2S inhibits neutrophil adhesion and activation in response to inflammatory stimuli and suppresses the release of the pro-inflammatory mediator tumor necrosis factor-alpha.[34,35] Other studies report that H_2S mediates pro-inflammatory effects by potentiating sulfide production in neutrophils[36] and mediating leukocyte activation.[37] Although granulocyte influx seems to be reduced by treatment with H_2S, literature is inconclusive on the contribution of neutrophil invasion to final myocardial infarct size and appears not to be a dominant factor.[38]

We show that treatment with H_2S protects against fibrosis at day 7 of reperfusion, as evidenced by reduced collagen deposition and fibronectin expression. Interestingly, the amount of necrosis differs between 10 and 100 ppm H_2S at day 1 of reperfusion, but this does not translate into differences in fibrotic area size after 7 days. This indicates that treatment with both concentrations of H_2S attenuate the onset of fibrosis. The prevention of fibrosis is in accordance with previous literature showing decreased cardiac remodelling and fibrosis in models of myocardial infarction and heart failure after H_2S treatment.[15,39,40] Although we find reduced fibrosis with both concentrations of H_2S, a balanced development of fibrosis remains essential. Suppressed fibrosis with no reduction in the extent of necrosis predisposes to infarct expansion and tissue rupture.[41] Since 10 ppm H_2S does not affect necrosis 1 day post-reperfusion, the anti-fibrotic effects at day 7 of reperfusion are not beneficial per se.

Another functional property of H_2S relates to the inhibition of ROS production, since the imbalance in redox status and oxidative stress contributes to fibrosis.[42] ROS-generating NOX2 and NOX4 are both increased after ischemic events in experimental models and their deficiency is protective in these models.[43,44] Seven days post-reperfusion, we found attenuated expression of NOX2 and NOX4 in both H_2S treated groups indicating less ROS production in vivo. We did not find an alteration of these genes at 1 day of reperfusion, which is in concordance with previous literature concerning NOX2.[45] Although it is not possible to distinguish whether these components originate from the myocardium or from phagocytes migrated into the myocardium, these results point towards increased oxidative stress in the infarcted heart, and a possible beneficial involvement for the effects of H_2S at the later time point. Furthermore, ROS production was markedly reduced in H_2S treated cardiomyoblasts in an *in vitro* model of

Antimycin induced oxidative stress, indicating direct scavenging or reduction in production of ROS by mitochondria. H_2S has direct scavenging effects on ROS, but also has indirect effects via activation of antioxidant mechanisms, such as increasing glutathione levels.[46,47] Another mechanism that could be involved is the capacity of H_2S to modulate cellular respiration, as the inhibition of mitochondrial respiration has been shown to protect against myocardial IRI by limiting ROS production in mitochondria.[48] Antioxidant effects of H_2S may be of critical importance for the treatment of myocardial IRI because oxidative stress plays a prominent role in the development of cardiac damage and remodeling.[42]

The effect of exogenous H_2S on blood pressure is still under debate. *In vivo* and *ex vivo* studies revealed conflicting responses to H_2S treatment.[49-53] The effects of H_2S on heart rate are also ambiguous; ranging from no change[54] to decreased heart rate in others.[50] Ufnal et al. noticed an increased heart rate upon NaHS infusion, however dependent on H_2S concentration in cerebrospinal fluid.[55] In additional support of this last view, suppression of H_2S production either pharmacologically[56] or genetically[57] leads to an increase in blood pressure. These opposing results might be attributable to differences in dose and route of administration.

In this study we show that 100 ppm of gaseous H_2S significantly lowers blood pressure and heart rate which might have affected cardiac workload and oxygen demand. Since we did not add a group with similar decrease in heart rate and blood pressure or a hypometabolic group with normal heart rate and blood pressure, we can not exclude this phenomenon to be responsible for the improved outcome. Aside from other protective effects of H_2S, it is thought that the reduced demand for oxygen during hypometabolism might be one of the protective mechanisms during ischemia based on the fact that oxygen availability and oxygen expenditure are more balanced. On the other hand the protective effects can not solely be explained by these effects since 10 ppm H_2S does not alter heart rate and blood pressure and has positive effects on several damage parameters. Another approach might be local delivery of H_2S by H_2S-donors thereby circumventing its systemic effects, which has previously been shown to be protective.[15] However, the highly volatile nature of H_2S and the associated difficulties in measuring this compound make it difficult to determine the exact dose and how long its effects endure, when given locally.

In conclusion, gaseous administration of H_2S protects the heart from IRI, likely through reduction of myocardial ROS production and the inhibition of inflammation, necrosis and fibrogenesis. Hypometabolism-inducing concentrations of H_2S seem to have additional protective effects on necrotic cell death shortly after ischemia. H_2S treatment might be of clinical use in myocardial ischemia or cardiac transplantation, where it could lead to reduced myocardial damage related to hypoxia.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude towards Marian Bulthuis and Susanne Veldhuis for their excellent technical support.

REFERENCES

1. Hausenloy DJ, Boston-Griffiths E, Yellon DM. (2012) Cardioprotection during cardiac surgery. Cardiovasc Res 94: 253-265.
2. Hori M, Nishida K. (2009) Oxidative stress and left ventricular remodelling after myocardial infarction. Cardiovasc Res 81: 457-464.
3. Hishikari K, Suzuki J, Ogawa M, Isobe K, Takahashi T, et al. (2009) Pharmacological activation of the prostaglandin E2 receptor EP4 improves cardiac function after myocardial ischaemia/reperfusion injury. Cardiovasc Res 81: 123-132.
4. Ojha N, Roy S, Radtke J, Simonetti O, Gnyawali S, et al. (2008) Characterization of the structural and functional changes in the myocardium following focal ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 294: H2435-43.
5. Yellon DM, Hausenloy DJ. (2007) Myocardial reperfusion injury. N Engl J Med 357: 1121-1135.
6. Szabo C. (2007) Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov 6: 917-935.
7. Wu N, Siow YL, O K. (2010) Ischemia/reperfusion reduces transcription factor Sp1-mediated cystathionine beta-synthase expression in the kidney. J Biol Chem 285: 18225-18233.
8. Xiaohui L, Junbao D, Lin S, Jian L, Xiuying T, et al. (2005) Down-regulation of endogenous hydrogen sulfide pathway in pulmonary hypertension and pulmonary vascular structural remodeling induced by high pulmonary blood flow in rats. Circ J 69: 1418-1424.
9. Aminzadeh MA, Vaziri ND. (2012) Downregulation of the renal and hepatic hydrogen sulfide (H_2S)-producing enzymes and capacity in chronic kidney disease. Nephrol Dial Transplant 27: 498-504.
10. Blackstone E, Morrison M, Roth MB. (2005) H_2S induces a suspended animation-like state in mice. Science 308: 518.
11. Bos EM, Leuvenink HG, Snijder PM, Kloosterhuis NJ, Hillebrands JL, et al. (2009) Hydrogen sulfide-induced hypometabolism prevents renal ischemia/reperfusion injury. J Am Soc Nephrol 20: 1901-1905.
12. Hill BC, Woon TC, Nicholls P, Peterson J, Greenwood C, et al. (1984) Interactions of sulphide and other ligands with cytochrome c oxidase. an electron-paramagnetic-resonance study. Biochem J 224: 591-600.
13. Kimura H. (2011) Hydrogen sulfide: Its production, release and functions. Amino Acids 41: 113-121.
14. Olson KR. (2011) The therapeutic potential of hydrogen sulfide: Separating hype from hope. Am J Physiol Regul Integr Comp Physiol 301: R297-312.
15. Calvert JW, Elston M, Nicholson CK, Gundewar S, Jha S, et al. (2010) Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. Circulation 122: 11-19.
16. Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, et al. (2007) Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci U S A 104: 15560-15565.
17. Minamishima S, Bougaki M, Sips PY, Yu JD, Minamishima YA, et al. (2009) Hydrogen sulfide improves survival after cardiac arrest and cardiopulmonary resuscitation via a nitric oxide synthase 3-dependent mechanism in mice. Circulation 120: 888-896.
18. Szabo G, Veres G, Radovits T, Gero D, Modis K, et al. (2011) Cardioprotective effects of hydrogen sulfide. Nitric Oxide 25: 201-210.
19. Haouzi P, Notet V, Chenuel B, Chalon B, Sponne I, et al. (2008) H_2S induced hypometabolism in mice is missing in sedated sheep. Respir Physiol Neurobiol 160: 109-115.
20. Li J, Zhang G, Cai S, Redington AN. (2008) Effect of inhaled hydrogen sulfide on metabolic responses in anesthetized, paralyzed, and mechanically ventilated piglets. Pediatr Crit Care Med 9: 110-112.
21. Bos EM, Snijder PM, Jekel H, Weij M, Leemans JC, et al. (2012) Beneficial effects of gaseous hydrogen sulfide in hepatic ischemia/reperfusion injury. Transpl Int 25: 897-908.
22. Damman J, Nijboer WN, Schuurs TA, Leuvenink HG, Morariu AM, et al. (2011) Local renal complement C3 induction by donor brain death is associated with reduced renal allograft function after transplantation. Nephrol Dial Transplant 26: 2345-2354.
23. Morrison ML, Blackwood JE, Lockett SL, Iwata A, Winn RK, et al. (2008) Surviving blood loss using hydrogen sulfide. J Trauma 65: 183-188.
24. Liu H, Bai XB, Shi S, Cao YX. (2009) Hydrogen sulfide protects from intestinal ischaemia-

reperfusion injury in rats. J Pharm Pharmacol 61: 207-212.

25. Fu Z, Liu X, Geng B, Fang L, Tang C. (2008) Hydrogen sulfide protects rat lung from ischemia-reperfusion injury. Life Sci 82: 1196-1202.
26. Jha S, Calvert JW, Duranski MR, Ramachandran A, Lefer DJ. (2008) Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: Role of antioxidant and antiapoptotic signaling. Am J Physiol Heart Circ Physiol 295: H801-6.
27. Sivarajah A, Collino M, Yasin M, Benetti E, Gallicchio M, et al. (2009) Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. Shock 31: 267-274.
28. Tokuda K, Kida K, Marutani E, Crimi E, Bougaki M, et al. (2012) Inhaled hydrogen sulfide prevents endotoxin-induced systemic inflammation and improves survival by altering sulfide metabolism in mice. Antioxid Redox Signal 17: 11-21.
29. Kida K, Yamada M, Tokuda K, Marutani E, Kakinohana M, et al. (2011) Inhaled hydrogen sulfide prevents neurodegeneration and movement disorder in a mouse model of parkinson's disease. Antioxid Redox Signal 15: 343-352.
30. Volpato GP, Searles R, Yu B, Scherrer-Crosbie M, Bloch KD, et al. (2008) Inhaled hydrogen sulfide: A rapidly reversible inhibitor of cardiac and metabolic function in the mouse. Anesthesiology 108: 659-668.
31. Blackstone E, Roth MB. (2007) Suspended animation-like state protects mice from lethal hypoxia. Shock 27: 370 -372.
32. Bracht H, Scheuerle A, Groger M, Hauser B, Matallo J, et al. (2012) Effects of intravenous sulfide during resuscitated porcine hemorrhagic shock*. Crit Care Med 40: 2157-2167.
33. Simon F, Giudici R, Duy CN, Schelzig H, Oter S, et al. (2008) Hemodynamic and metabolic effects of hydrogen sulfide during porcine ischemia/reperfusion injury. Shock 30: 359-364.
34. Fiorucci S, Antonelli E, Distrutti E, Rizzo G, Mencarelli A, et al. (2005) Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. Gastroenterology 129: 1210 -1224.
35. Hu LF, Wong PT, Moore PK, Bian JS. (2007) Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia. J Neurochem 100: 1121-1128.
36. Mitsuhashi H, Yamashita S, Ikeuchi H, Kuroiwa T, Kaneko Y, et al. (2005) Oxidative stress-dependent conversion of hydrogen sulfide to sulfite by activated neutrophils. Shock 24: 529-534.
37. Zhang H, Bhatia M. (2008) Hydrogen sulfide: A novel mediator of leukocyte activation. Immunopharmacol Immunotoxicol 30: 631-645.
38. Baxter GF. (2002) The neutrophil as a mediator of myocardial ischemia-reperfusion injury: Time to move on. Basic Res Cardiol 97: 268-275.
39. Qipshidze N, Metreveli N, Mishra PK, Lominadze D, Tyagi SC. (2012) Hydrogen sulfide mitigates cardiac remodeling during myocardial infarction via improvement of angiogenesis. Int J Biol Sci 8: 430-441.
40. Wang X, Wang Q, Guo W, Zhu YZ. (2011) Hydrogen sulfide attenuates cardiac dysfunction in a rat model of heart failure: A mechanism through cardiac mitochondrial protection. Biosci Rep 31: 87-98.
41. Ichihara S, Senbonmatsu T, Price E,Jr, Ichiki T, Gaffney FA, et al. (2002) Targeted deletion of angiotensin II type 2 receptor caused cardiac rupture after acute myocardial infarction. Circulation 106: 2244-2249.
42. Ma L, Gul R, Habibi J, Yang M, Pulakat L, et al. (2012) Nebivolol improves diastolic dysfunction and myocardial remodeling through reductions in oxidative stress in the transgenic (mRen2) rat. Am J Physiol Heart Circ Physiol 302: H2341-51.
43. De Silva TM, Brait VH, Drummond GR, Sobey CG, Miller AA. (2011) Nox2 oxidase activity accounts for the oxidative stress and vasomotor dysfunction in mouse cerebral arteries following ischemic stroke. PLoS One 6: e28393.
44. Kleinschnitz C, Grund H, Wingler K, Armitage ME, Jones E, et al. (2010) Post-stroke inhibition of induced NADPH oxidase type 4 prevents oxidative stress and neurodegeneration. PLoS Biol 8: 10.1371/journal.pbio.1000479.
45. Fukui T, Yoshiyama M, Hanatani A, Omura T, Yoshikawa J, et al. (2001) Expression of p22-phox and gp91-phox, essential components of NADPH oxidase, increases after myocardial infarction. Biochem Biophys Res Commun 281: 1200-1206.
46. Kimura Y, Goto Y, Kimura H. (2010) Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. Antioxid Redox Signal 12: 1-13.

47. Kimura Y, Kimura H. (2004) Hydrogen sulfide protects neurons from oxidative stress. FASEB J 18: 1165-1167.

48. Chen Q, Moghaddas S, Hoppel CL, Lesnefsky EJ. (2006) Reversible blockade of electron transport during ischemia protects mitochondria and decreases myocardial injury following reperfusion. J Pharmacol Exp Ther 319: 1405-1412.

49. Ali MY, Ping CY, Mok YY, Ling L, Whiteman M, et al. (2006) Regulation of vascular nitric oxide in vitro and in vivo; a new role for endogenous hydrogen sulphide? Br J Pharmacol 149: 625-634.

50. Dawe GS, Han SP, Bian JS, Moore PK. (2008) Hydrogen sulphide in the hypothalamus causes an ATP-sensitive K+ channel-dependent decrease in blood pressure in freely moving rats. Neuroscience 152: 169-177.

51. Yan H, Du J, Tang C. (2004) The possible role of hydrogen sulfide on the pathogenesis of spontaneous hypertension in rats. Biochem Biophys Res Commun 313: 22-27.

52. Zhao W, Zhang J, Lu Y, Wang R. (2001) The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J 20: 6008-6016.

53. Kohn C, Schleifenbaum J, Szijarto IA, Marko L, Dubrovska G, et al. (2012) Differential effects of cystathionine-gamma-lyase-dependent vasodilatory H_2S in periadventitial vasoregulation of rat and mouse aortas. PLoS One 7: e41951.

54. Johansen D, Ytrehus K, Baxter GF. (2006) Exogenous hydrogen sulfide (H_2S) protects against regional myocardial ischemia-reperfusion injury--evidence for a role of K ATP channels. Basic Res Cardiol 101: 53-60.

55. Ufnal M, Sikora M, Dudek M. (2008) Exogenous hydrogen sulfide produces hemodynamic effects by triggering central neuroregulatory mechanisms. Acta Neurobiol Exp (Wars) 68: 382-388.

56. Lavu M, Bhushan S, Lefer DJ. (2011) Hydrogen sulfide-mediated cardioprotection: Mechanisms and therapeutic potential. Clin Sci (Lond) 120: 219-229.

57. Yang G, Wu L, Jiang B, Yang W, Qi J, et al. (2008) H_2S as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. Science 322: 587-590.

5

CYSTATHIONINE γ-LYASE PROTECTS AGAINST RENAL ISCHEMIA / REPERFUSION BY MODULATING OXIDATIVE STRESS

EM Bos
R Wang
PM Snijder
M Boersema
J Damman
M Fu
J Moser
JL Hillebrands
RJ Ploeg
G Yang
HGD Leuvenink
H van Goor

Published in the Journal of the American Society of Nephrology

Reference: J Am Soc Nephrol, 2013 Apr;24(5):759-70
Digital object identifier (DOI): 10.1681/ASN.2012030268

ABSTRACT

Hydrogen sulfide (H_2S) is now recognized as the third endogenous gasotransmitter, with similar physiological functions to nitric oxide and carbon monoxide. In this study, we investigated whether endogenous production of H_2S by cystathionine γ-lyase (CSE) has protective effects – similar to treatment with exogenous H_2S – in a renal ischemia setting. We found that CSE is abundantly expressed in the kidney, while CSE knockout animals had markedly reduced renal production of H_2S. CSE deficiency was associated with increased damage and mortality after renal ischemia/reperfusion injury (IRI). Treatment with exogenous H_2S rescued CSE knockout mice from mortality and injury associated with renal ischemia. In addition, overexpression of CSE in vitro reduced the amount of reactive oxygen species produced during stress. Finally, CSE expression in renal transplant donors was positively associated with superior outcome after transplantation. These results indicate a role for CSE as a modulator of oxidative stress through the production of H_2S.

INTRODUCTION

In recent years, the fundamental physiological role of hydrogen sulfide (H_2S) has gradually been uncovered. H_2S is now acknowledged as the third endogenously produced gaseous signaling molecule, in addition to nitric oxide (NO) and carbon monoxide (CO)[1]. H_2S is generated from the amino acid L-cysteine by three distinct enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MPST)[2]. In the cardiovascular system, CSE is the most abundantly expressed protein, and is responsible for the majority of endogenous H_2S production3. The physiologic properties of endogenously produced H_2S are remarkably similar among the different gasotransmitters. H_2S functions as an endothelial-cell derived relaxing factor (EDRF) similar to NO[4]. Accordingly CSE deficient mice ($CSE^{-/-}$) develop hypertension[3], analogous to mice lacking endothelial nitric oxide synthase (eNOS)[5] and $CBS^{+/-}$ mice[6]. In addition, endogenously produced H_2S is involved in cellular proliferation[7], angiogenesis[8], inflammation[9,10] and regulation of protein activity through S-sulfhydration[11-13].

Exogenous treatment with H_2S can induce a reversible hypometabolic, hibernation-like state[14]. The proposed mechanism behind H_2S -induced hypometabolism is through the reduction of mitochondrial activity by reversible binding to cytochrome c oxidase, the terminal enzyme in the electron transport chain[15]. During hypoxia, H_2S treatment ameliorates the reduction in the function and integrity of mitochondria[16-18]. The lower demand for oxygen during hypometabolism can protect animals from hypoxia or shock, and organs from ischemia/reperfusion injury[16,17,19,20]. Exogenous H_2S can play a detoxifying role during oxidative stress by directly scavenging reactive oxygen/nitrogen species (ROS/RNS) as well as increasing the formation of the antioxidant glutathione (GSH)[21,22].

In this study we found that CSE deficiency in mice led to reduced renal H_2S production and was associated with increased mortality and severity of damage after renal ischemia/reperfusion injury. Administering exogenous H_2S rescued these mice from mortality and injury associated with renal ischemia. Our in vivo experiments together with in vitro cell studies in which CSE is overexpressed highlight the fundamental role CSE has in regulating the amount of ROS associated with hypoxic stress. In addition, we notably found that CSE mRNA and protein levels were shown to be associated with kidney outcome after transplantation. Together, our data reveal CSE as a modulator of oxidative stress induced following renal ischemia.

RESULTS

Localization of CSE in control human renal tissue

In human renal tissue, CSE protein was localized to glomeruli and tubulo-interstitium. Glomeruli were homogenously positive for CSE. Non-endothelial (CD31-negative) glomerular cells showed positive staining in addition to (CD31-positive) endothelial cells, indicating expression in mesangial cells and/or podocytes. (Figure 1A). CSE was seen in tubular epithelium – proximal as well as distal (Figure 1B), peritubular capillaries (Figure 1C), and vascular endothelium (Figure 1D). The protein was observed in an unidentified intracellular staining pattern, showing a distinct line-like staining in the cytosolic compartments of tubules and non-glomerular endothelial

cells (Figure 1B). TissueFAXS analysis showed that 75% of renal cells were positive for CSE (Figure 1E). Moreover, when we investigated endothelial cells, we found that 87% of endothelium was positive for CSE (Figure 1F). Transplant biopsies showed similar localization and staining pattern as the control renal tissue (data not shown).

CSE mRNA levels are modulated after renal ischemia in rats

The expression of CSE mRNA was modulated after ischemia in control rat kidneys. Early time points show an increase in renal CSE mRNA (90 minutes), after which expression decreased to significantly lower levels compared to basal levels (Figure 2A). At 1, 2 and 4 days after ischemia mRNA levels were decreased after which mRNA levels normalized to basal values after 9 days and afterwards (Figure 2A). The expression of CBS was modulated in a temporally similar pattern (Figure 2B).

CSE but not CBS mRNA expression levels were altered during the transplant process

CSE mRNA levels were significantly increased after reperfusion ($p<0.01$) (Figure 2C). Expression levels were (Median [range]): procurement 0.67 [0.03-1.66], pre-implantation 0.41 [0.08-1.75], reperfusion 0.93 [0.15-3.65]. CBS mRNA levels were not modulated during the transplant process (Figure 2D): Procurement 0.20 [0.001-1.99], pre-implantation 0.23 [0.007-1.09], reperfusion 0.20 [0.02-0.86].

Modulation of CSE protein levels during the transplant process

CSE immunofluorescence was performed on a limited number of renal transplant biopsies (n=27). No differences in protein levels were detected between pre-implantation and reperfusion time points (Figure 2E).

CSE deficient mice have reduced renal H_2S production

Kidneys from untreated wildtype (WT) and CSE knockout ($CSE^{-/-}$) animals were examined to confirm that CSE deficiency caused reduced production of H_2S in renal tissue. Renal weight did not significantly differ between WT and $CSE^{-/-}$ mice (data not shown). $CSE^{-/-}$ mice had a 91% reduction in renal H_2S production compared to WT mice ($p<0.05$, Figure 3A).

Increased mortality after bilateral renal ischemia in CSE deficient mice

Mortality after IRI was significantly higher in the $CSE^{-/-}$ animals, where 35% (5/14) of animals died within the first 24 hours, while WT animals showed 0% (0/14) mortality during this period ($p<0.05$, Figure 3B). $CSE^{-/-}$ animals could be rescued by pretreatment with NaHS, with 11% (1/9) mortality compared to 0% (0/9) in WT animals (p=ns, Figure 3C).

CSE deficient mice display more severe kidney damage and decreased renal function after IRI compared to WT mice

No necrosis was detected in kidneys of sham-operated mice, while all kidneys subjected to IR showed tubular necrosis in the cortico-medullary transition area. $CSE^{-/-}$ animals showed 60% higher levels of necrosis compared to WT animals ($p<0.01$, Figure 3D, representative examples in Figure 3F, representative photomicrographs in Supplementary figure 1). The amount of necrosis was reduced by treatment with NaHS regardless of genotype. Renal function as measured by plasma creatinine levels showed a similar pattern, with a 51% higher level of creatinine in $CSE^{-/-}$

Figure 1

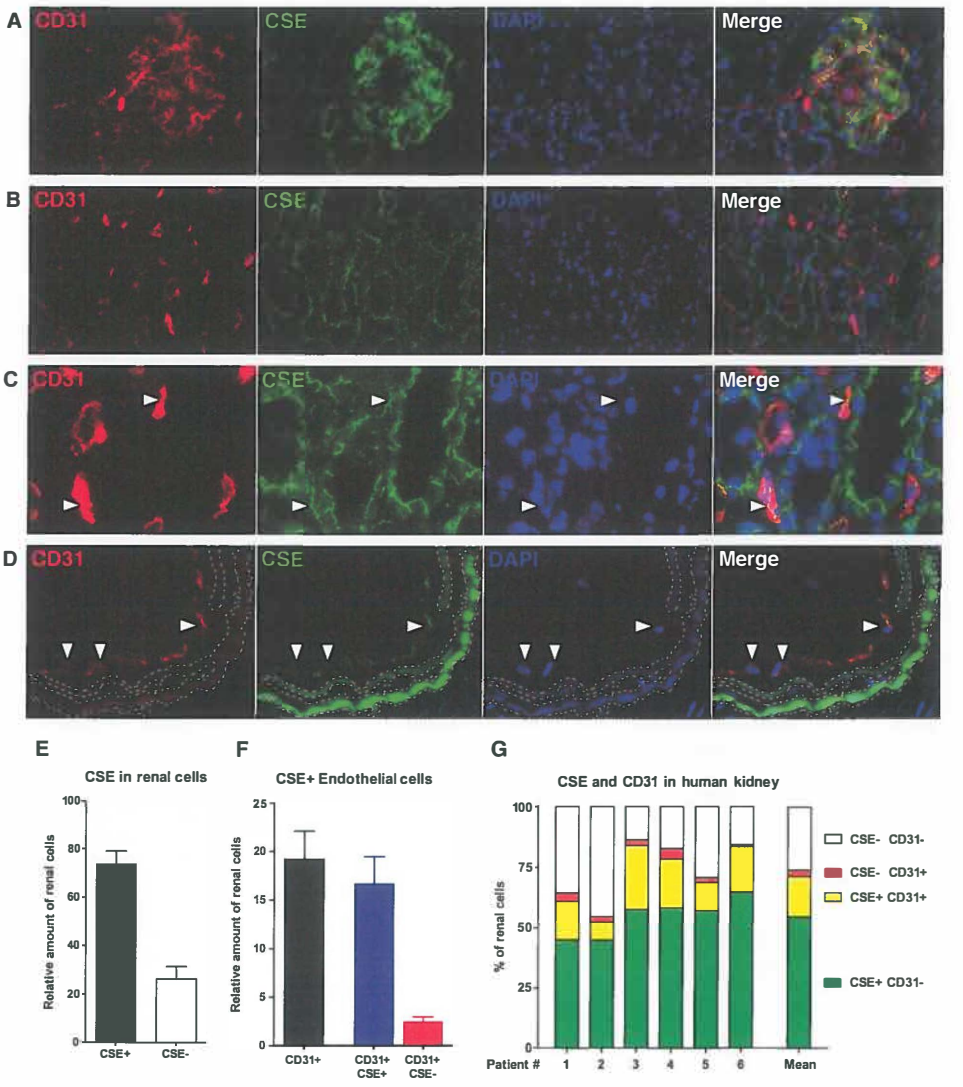


Figure 1 – Localization of CSE in control human renal tissue.
Representative examples of the localization of CSE (green) and the colocalization with CD31 positive cells (red). CSE is localized in (A) the glomerulus (endothelial and mesangial cells), (B) tubules, (C) peritubular capillaries (colocalization of CSE with CD31 is seen as yellow in the merged images, and is marked by white arrowheads. Image is a magnification from (B)) and (D) vascular endothelial cells (white arrowheads). In (D), aspecific autofluorescence of the elastic laminae is demarcated in dashed lines. CSE is expressed in proximal and distal renal tubules (A,B,C) in an unidentified subcellular staining pattern. Original magnification: 630X. (E) TissueFAXS analysis of the total amount of CSE-expressing cells in the kidney of six control kidneys, indicating that 75% of renal cells express CSE. (F) Of all renal endothelial cells, the large majority express CSE. (G) The variation between control patients in the abundance of CSE and CD31 expressing cells.

Figure 2

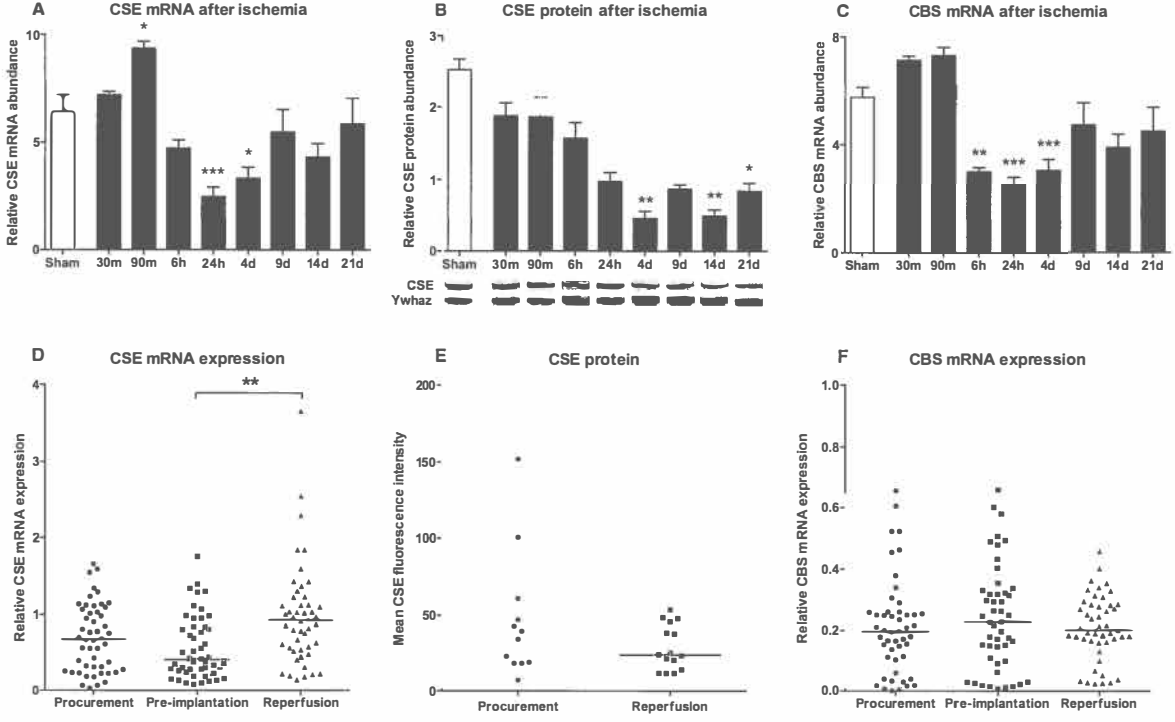


Figure 2 – Time-course expression of CSE/CBS after ischemia/reperfusion.

Renal expression of (A) CSE and (C) CBS at various time points in the first 21 days after renal ischemia in rats (n=5-6 animals per group). CSE mRNA is increased in the acute stage, and both are reduced in the medium-long term, and normalized after 9 days (* - p<0.05; ** - p<0.01; *** - p<0.001). (B) CSE protein abundance as measured by Western blot, showing significantly reduced CSE protein at 4, 14 and 21 days (* - p<0.05; ** - p<0.01, n=3-5 animals per group). Under each bar representative protein bands, all from the same blot. Expression of CSE mRNA (D) and CBS mRNA (F) in human donor biopsies at different time points during the transplant process. CSE expression at reperfusion is significantly increased compared to pre-implantation (** - p<0.01). (E) No significant difference in CSE protein levels between procurement and reperfusion time points in human renal transplantation was found.

animals compared to WT animals after IRI. Pretreatment with NaHS significantly improved renal function in $CSE^{-/-}$ animals ($p<0.001$, Figure 3E). In sham-operated animals creatinine levels were similar, indicating that renal function in untreated $CSE^{-/-}$ is not impaired (Figure 3E).

Renal inflammation after renal ischemia/reperfusion

Granulocyte influx as measured by Ly-6G immunohistochemistry showed an increase in granulocytes after ischemia in both WT and $CSE^{-/-}$ animals, which was abrogated by NaHS treatment regardless of genotype. There was no difference in the amount of granulocytes between WT and $CSE^{-/-}$ animals (Figure 3G).

CSE mice display increased levels of DNA damage after ischemia/reperfusion

In order to determine the extent of oxidative damage after IRI we carried out immunofluorescence staining for γH2AX, a marker for DNA double strand breaks (DSBs) on kidney sections from CSE deficient and WT mice. γH2AX plays an important role in the DNA damage response and is necessary for the initial rapid phase of DSB repair. pγH2AX is a proven marker of oxidative stress23. We found phosphorylated γH2AX positive cells in the cortical tubular cells of mice after IRI the amount of positive cells was significantly higher in $CSE^{-/-}$ mice compared to WT mice (Figure 4A). Pretreatment with NaHS reduced the amount of pγH2AX positive cells in both WT and CSE deficient mice but did not reduce the amount of positive cells to sham injury levels (Figure 4A).

Proliferation after renal ischemia/reperfusion

Ki67 staining indicated that $CSE^{-/-}$ animals did not have increased proliferation after IRI (Figure 4B). The amount of Ki67 positive nuclei was significantly increased after NaHS treatment in combination with IRI in $CSE^{-/-}$ animals, but not in the WT animals (Figure 4B).

Renin is not differentially expressed in wildtype and $CSE^{-/-}$ mice

Immunohistochemistry for renin showed its expression in the juxtaglomerular cells. When the amount of glomeruli with renin-positive juxtaglomerular cells were counted, no significant differences between the groups were found (Figure 5A). The number of renin-positive juxtaglomerular cells per glomerulus did not differ between groups (Figure5B). The lack of renal H_2S production in $CSE^{-/-}$ mice does not affect renin levels, nor does the treatment of wildtype or $CSE^{-/-}$ animals with NaHS. IRI also does not affect renin levels 24 hours after ischemia.

Cell viability, EGFP fluorescence and CSE protein abundance after transfection

Transfection with the pIRES2-EGFP or pIRES2-EGFP-CSE plasmids did not affect viability of HEK293 cells between 1 and 4 days after transfection (Figure 6A). Both plasmids increased the amount of EGFP fluorescence between 1 and 5 days after transfection, reaching a peak between 3-4 days (Figure 6B). The amount of EGFP fluorescence was not significantly different between the two plasmids. Western blot at 72 hours after transfection showed no effect of mock-transfection with pIRES2-EGFP on CSE protein, while the pIRES2-EGFP-CSE plasmid induced a 6-fold increase in the band density for CSE (Figure 6C). The amount of H_2S in the supernatant medium of the pIRES2-EGFP-CSE transfected cells did not significantly differ from the H_2S levels in the control and pIRES2-EGFP transfected groups (Figure 6D).

Figure 3

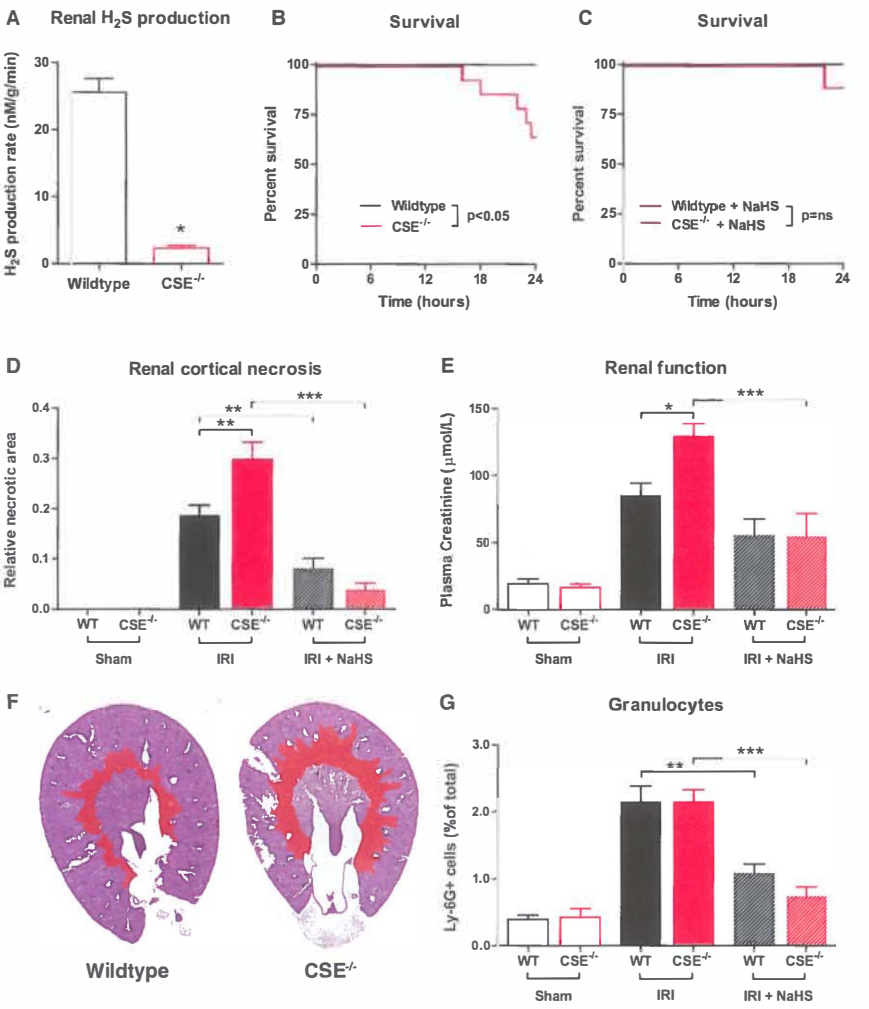


Figure 3 – Renal H_2S production, survival, renal function, necrosis and inflammation in CSE$^{-/-}$ mice after ischemia/reperfusion.

(A) Renal H_2S production rate is significantly lower in CSE$^{-/-}$ mice compared to WT mice (* - p<0.05, n=4 per group). (B) Animal survival after renal IRI is impaired in the CSE$^{-/-}$ animals (p<0.05, n=14 per group). (C) Treatment with NaHS rescues CSE$^{-/-}$ mice from IRI-induced mortality (p=ns, WT: n=7; CSE$^{-/-}$: n=9). (D) The amount of renal cortical necrosis is significantly higher in CSE$^{-/-}$ mice (n=9) compared to WT (n=14), while pretreatment with NaHS reduces necrosis in both WT (n=7) as well as CSE$^{-/-}$ mice (n=8) (** - p<0.01; *** - p<0.001, WT Sham: n=9; CSE$^{-/-}$ Sham: n=8). (E) Plasma creatinine levels indicate reduced renal function after ischemia in CSE$^{-/-}$ (n=9) compared to WT mice (n=14). NaHS treatment protected CSE$^{-/-}$ mice (n=8) from the IRI-induced decline in renal function (* - p<0.05; *** - p<0.001, WT Sham: n=9; CSE$^{-/-}$ Sham: n=8). (F) Representative examples of PAS-stained coronal renal sections from WT and CSE$^{-/-}$ animals with the necrotic area artificially colored red. (G) Influx of Ly-6G-positive granulocytes was not affected by CSE deficiency (CSE$^{-/-}$: n=9, WT: n=14). Granulocyte influx was reduced in both WT (n=7) and CSE$^{-/-}$ animals (n=8) after NaHS pretreatment (** - p<0.01; *** - p<0.001, WT Sham: n=9; CSE$^{-/-}$ Sham: n=8).

Figure 4

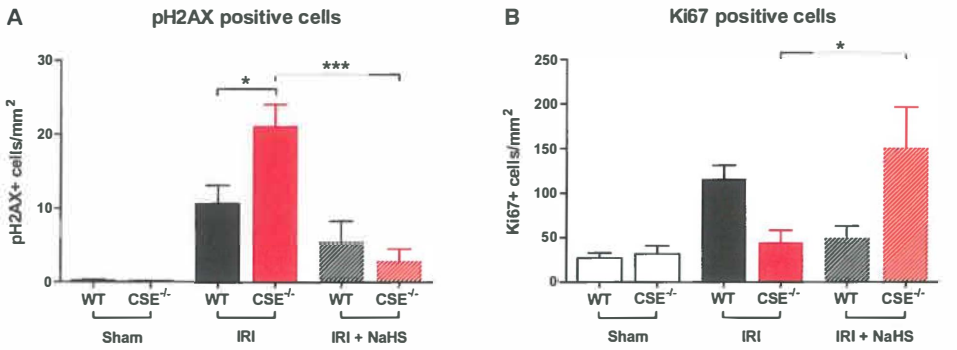


Figure 4 – Expression of pγH2AX and Ki67 in WT and CSE$^{-/-}$ animals after ischemia/reperfusion. (A) Abundance of the phosphorylated DNA-repair protein γH2AX is significantly higher in CSE$^{-/-}$ animals after IRI (n=9) compared to WT animals (n=14), indicating an increased amount of DNA double strand breaks, possibly related to oxidative DNA damage. (B) After IRI, the expression of the nuclear proliferation marker Ki67 is not increased in CSE$^{-/-}$ animals (n=9) compared to WT (n=14), but significantly increased after NaHS pretreatment and IRI combined (n=8, WT Sham: n=9; CSE$^{-/-}$ Sham: n=8).

Effects of exogenous NaHS and CSE overexpression on mitochondrial and overall superoxide

Antimycin A-induced oxidative stress as measured by DHE fluorescence in HEK293 cells was concentration dependently reduced by treatment with NaHS. Antimycin induced a 48 fold increase in DHE fluorescence which was significantly attenuated by NaHS at 10 µM, 100 µM and 1 mM (Figure 6E). Mitochondrial superoxide production as assessed by MitoSOX fluorescence showed a similar pattern, with a 13.5 fold increase in fluorescence, which was significantly lower when treated with 10 µM, 100 µM or 1 mM NaHS (Figure 6G).

Treatment with Antimycin A showed an increase in DHE (Figure 6F) and MitoSOX (Figure 6H) fluorescence in all groups. Fluorescence intensity was similar in control and pIRES2-EGFP transfected groups, while cells overexpressing CSE showed a significantly reduced amount of fluorescence. The reduction was 60% when measured using DHE (Figure 6F, red bar), and 74% using MitoSOX (Figure 6H, red bar). Treatment with 1 mM of NaHS reduced the amount of fluorescence of both probes in all groups except the CSE overexpressing cells loaded with MitoSOX (Figure 6H, red bars).

Association of CSE and CBS mRNA expression pre-transplantation and renal function after transplantation

CSE mRNA levels at organ procurement were positively associated with renal function 14 days after transplantation, as measured by glomerular filtration rate (GFR). Linear regression analysis showed that the association significantly deviated from zero (p=0.007), and the goodness of fit (R^2) was 0.3146 (Figure 7A). Relative CBS mRNA levels did not associate with renal function after transplantation, showing no significant deviation from zero (p=0.16) and low goodness of fit with an R^2 of 0.0262 (Figure 7B).

Figure 5

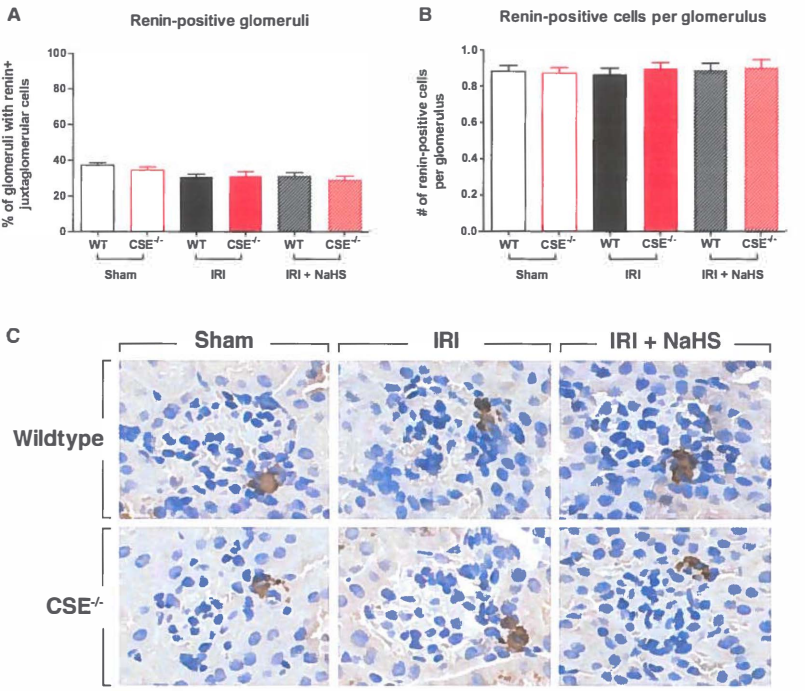


Figure 5 - No difference in renin expression between genotypes or between treatment groups. (A) Percentage of glomeruli which had renin-positive juxtaglomerular cells and (B) amount of renin-positive juxtaglomerular cells per glomerulus in the different groups did not differ. (C) Representative examples of glomeruli with renin-positive juxtaglomerular cells. Original magnification 200x.

Figure 6 [Opposite page] (A) Cell viability was not affected by transfection with pIRES2-EGFP or pIRES2-EGFP-CSE vectors. (B) EGFP fluorescence intensity after transfection, showing peak values between 72-96 hours after transfection with both control and CSE vector. (C) CSE protein as measured by western blot showed an increase of 6x after 72 hours after transfection with pIRES2-EGFP-CSE vector (*** - p<0.001), while there was no increase in CSE expression in the pIRES2-EGFP transfected cells compared to controls (representative bands from the same gel are shown). (D) No differences in supernatant H_2S were measured. (E) Antimycin induced cytoplasmatic ROS production as measured by DHE fluorescence was significantly and concentration dependently attenuated by treatment with NaHS (### - p<0.001 vs -NaHS, -Antimycin; * - p<0.05; *** - p<0.001 vs -NaHS, +Antimycin). (F) DHE fluorescence is not affected by transfection with the pIRES2-EGFP vector, but significantly reduced by transfection with pIRES2-EGFP-CSE. Treatment with NaHS further reduces DHE fluorescence intensity in all groups (* - p<0.05; *** - p<0.001). (G) Mitochondrial superoxide production, as measured with the fluorescent MitoSOX probe, is significantly and concentration dependently reduced by NaHS treatment (# - p<0.05; ### - p<0.001 vs -NaHS, -Antimycin; ** - p<0.01 vs -NaHS, +Antimycin). (H) pIRES2-EGFP transfection does not affect MitoSOX fluorescence after antimycin treatment, while pIRES2-EGFP-CSE transfection significantly reduces the amount of mitochondrial superoxide produced (* - p<0.05; *** - p<0.001). Addition of NaHS did not produce significant additional effects in the pIRES2-EGFP-CSE transfected cells. Data are representative of at least three independent experiments. ►

Figure 6

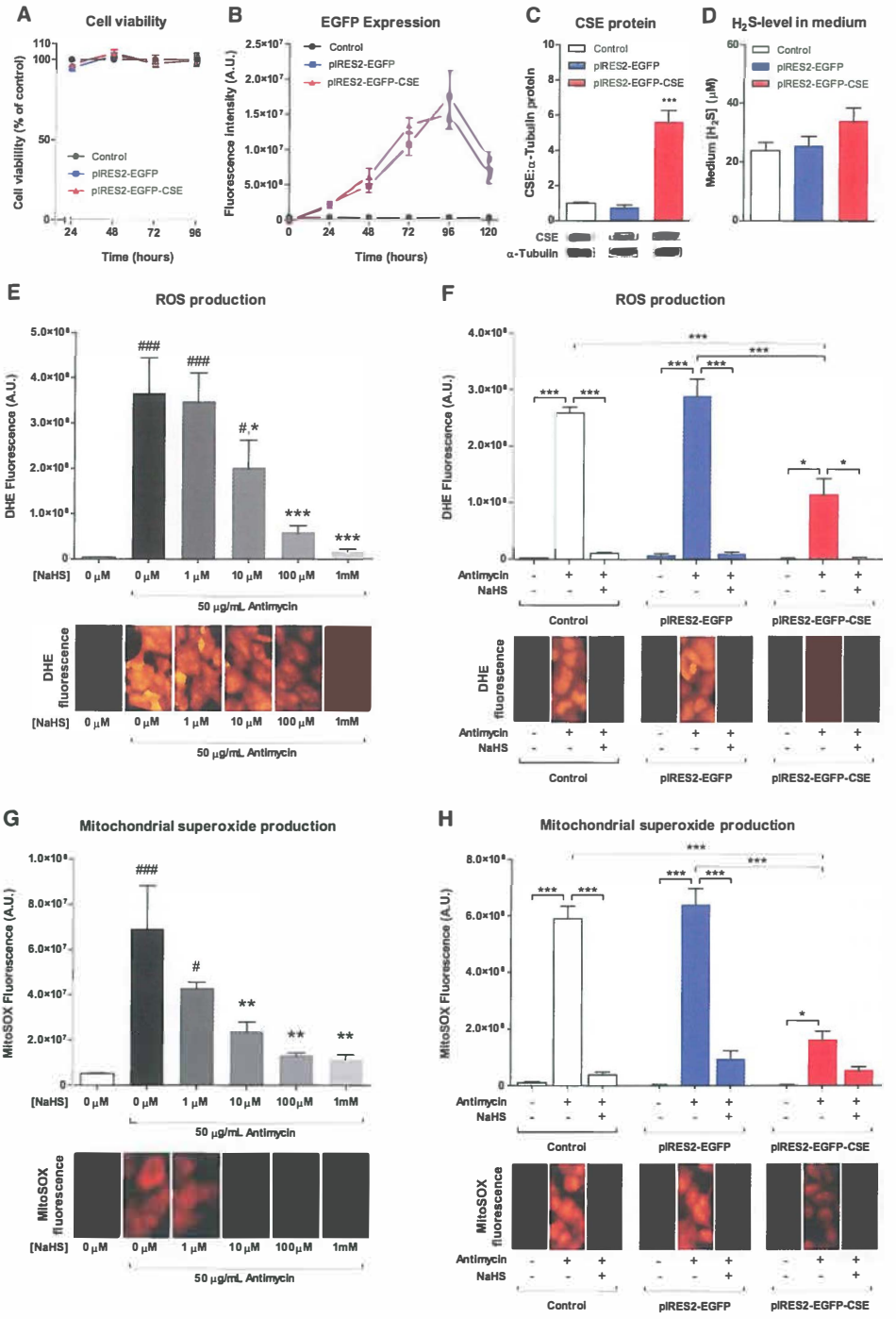


Figure 6 – CSE overexpression in vitro and production of ROS in HEK293 cells.

Figure 7

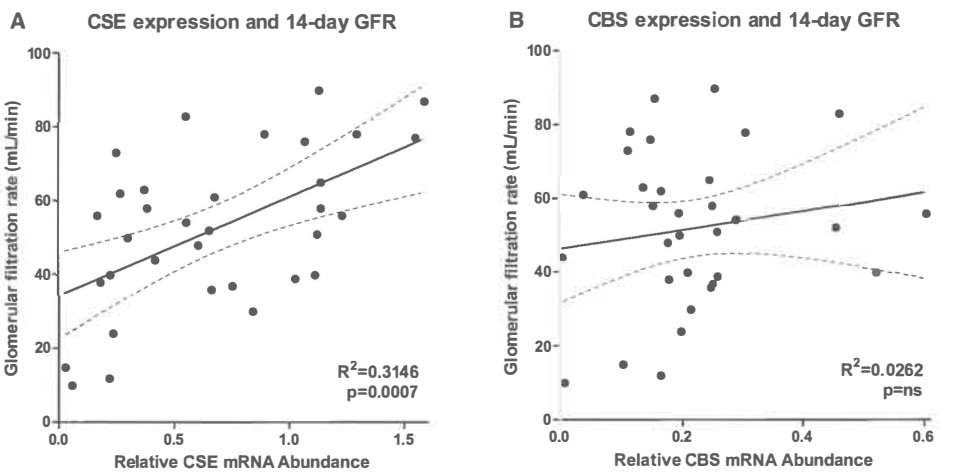


Figure 7 - Association between renal CSE/CBS mRNA and outcome after human renal transplantation. (A) Renal CSE mRNA level at organ procurement is associated with the glomerular filtration rate at 14 days after transplantation, with higher expression associating with better renal function (R^2=0.315, p<0.001, n=33). Renal CBS levels at procurement are not associated with outcome after renal transplantation (R^2=0.026, p=ns, n=32). Lines denote best fit, with the dashed lines representing the 95% confidence interval for best fit.

DISCUSSION

Over the past decades, the perceived image of H_2S has transformed from that of a dangerously toxic molecule to that of an endogenously produced gas affecting many physiological processes. The similarities between H_2S and the other gasotransmitters are numerous[1,2]. In this study, we show that the endogenous production of H_2S reduces the damage associated with renal ischemia/reperfusion injury. In previous papers, others and we have previously demonstrated the highly protective effects of exogenously applied H_2S treatment in similar models of ischemia[16,17,19,24-27]. From the present study it now becomes clear that after renal hypoxic stress, CSE functions as an endogenous mediator of the antioxidant response, most likely through the production of H_2S.

This study shows that CSE is abundantly present in the normal human kidney, with widespread expression in the tubules. We found that the majority of renal tubules stain positive for CSE, which aligns with a previous study indicating that CSE activity was found in all segments of the renal tubule, with the highest activity in the proximal straight and distal tubules[28]. The large majority of renal endothelial cells express CSE. In CSE deficient mice, renal H_2S production was reduced by more than 90%, indicating that CSE is the most essential H_2S producing enzyme in the kidney.

There was a clear association between the absence of CSE expression in the $CSE^{-/-}$ mice and an increase in renal damage after IRI. Mortality, renal failure and tubular necrosis were significantly increased when renal H_2S production was low. Treatment with exogenous NaHS rescued these animals from death and renal failure. The absence of CSE expression did not influence the

Table 1 - Characteristics of the human kidney donors analyzed in the different analyses performed in this paper.

Sex (Male:Female)	17:33
Age, years (Mean [Range])	49 [16-70]
Donor type (Living:Brain dead)	20:30
Cold ischemia time, minutes (Mean [Range])	742 [112-2091]
Warm ischemia time, minutes (Mean [Range])	39 [13-91]
Delayed graft function	11
Rejection (1 year)	9

inflammatory response. This indicates that endogenous production of H_2S does have the anti-inflammatory effects that the concentrations afforded by exogenous H_2S treatment has in this model[16]. Others have shown the inhibitory effects of endogenously produced H_2S by CSE on leukocyte adhesion[9]. We have no indication of the actual plasma concentrations and, perhaps more relevant, the intracellular concentrations caused by CSE/CBS/MPST activity relative to those brought by exogenous H_2S. This means that, in this model, the concentrations needed to produce anti-inflammatory effects might be too high to be reached by endogenous H_2S production. The known inhibitory effects of H_2S on inflammatory processes are likely absent in the $CSE^{-/-}$ mice, so increased influx of leukocytes was expected in this model, especially with the increased renal damage in the $CSE^{-/-}$ mice. The results indicate that CSE deficiency can also influence the inflammatory response to injury through an unknown mechanism.

Excessive generation of ROS following injury damages proteins, DNA, mitochondria and lipids and can stimulate the immune system leading to organ damage[29]. Hydroxyl radicals can also react with nearby tissues, resulting in cellular DNA damage[30]. Phosphorylated γH2AX is a well-known marker of DNA damage and, in particular, of DSBs[31]. Phosphorylated γH2AX is the first step in recruiting and localizing DNA repair proteins. We found more DSBs as detected by γH2AX staining in the kidneys of CSE mice after IRI compared to WT. Pretreatment of NaHS was associated with reduced amounts of DNA damage together with all the other parameters of renal injury. Our present results show severe DNA damage in $CSE^{-/-}$ mice was associated with a reduced but not significant number of Ki67 positive cells (hence; cell proliferation) when compared to WT mice.

Proliferation is affected by CSE, as Ki67 immunofluorescence showed. CSE activity or the availability of H_2S seems to be a necessity for the induction of proliferation in this model, as IRI by itself does not induce Ki67 expression in $CSE^{-/-}$ animals, but the combination of IRI and NaHS treatment induces high expression of this protein. Interestingly, treatment of wildtype animals with NaHS during IRI seems to inhibit proliferation, which correlates with the amount of renal cell damage in this experiment, indicating that the protective effects seen in wildtype animals are not due to an increase in regeneration. These results contrast earlier work, where CSE deficiency caused overproliferation in vascular smooth muscle cells[7].

Since $CSE^{-/-}$ animals develop higher blood pressure compared to wildtype after 6 weeks (a rise of about 10 mmHg at 7 weeks)[3], the differences between groups could be related to differences

in renal perfusion before or after the ischemic event. To investigate this, we measured renin expression in the juxtaglomerular apparatus (Figure 5 A-C). We found no differences between wildtype and $CSE^{-/-}$ mice, nor between IRI and NaHS treated groups. This indicates that there were no large differences in renal perfusion between the groups, and that the increase in renal damage after IRI in the $CSE^{-/-}$ animals, nor the protective effects of NaHS can be explained by altered perfusion. Ming et al. recently showed that H_2S can inhibit renin expression in a 2K1C model, but it did not affect expression in sham or unclipped kidneys[32]. We noted no effect of NaHS on renin expression, suggesting that H_2S can inhibit an increase in renin levels, but does not affect normal renin levels. However, renin protein levels have to be regarded as a crude marker for renal perfusion, for which changes van can only be detected with large perfusion differences.

In vitro experiments showed that exogenous H_2S treatment concentration-dependently reduced the amount of intracellular ROS production when stimulated with Antimycin. A significant effect was reached at low concentrations. Overexpression of CSE showed a reduction in oxidative stress that was similar to ~10 μM of NaHS. This concentration is close to the most quoted physiological range of 30-300 μM in human serum, but the actual physiological concentration in mammals is still up for discussion[33]. The addition of 1 mM NaHS showed a further reduction in DHE fluorescence, which might indicate that overexpression of CSE did not induce supraphysiological concentrations in this particular model. The fact that we did not find a significant increase in supernatant H_2S concentration after CSE overexpression is not unexpected, since H_2S rapidly evaporates from solution at physiological pH[34] or from serum[33].

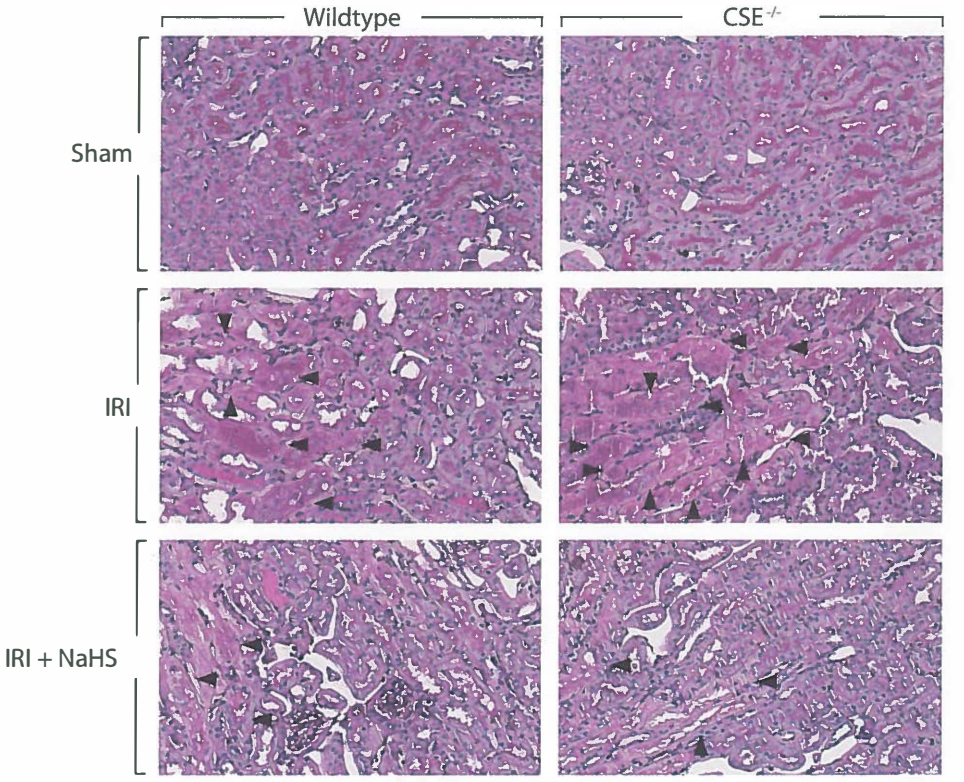
Whether H_2S directly scavenges ROS, increases the intracellular GSH levels or reduces the amount of ROS produced through modulation of mitochondrial ROS production was not assessed by this study. H_2S can exert all these effects[2], so the protective and antioxidant effects demonstrated here could be the result of multiple mechanisms acting simultaneously. One previous study assessed the effects of CSE overexpression in a murine model of myocardial infarction. Myocardium-specific overexpression of CSE nearly doubled H_2S production and reduces myocardial infarct size by 47%[17]. Other studies have shown the effects of inhibitors of CSE and CBS in ischemic models, but the specificity of these inhibitors is subject to discussion[35]. Most studies using these inhibitors imply protective effects afforded by endogenous H_2S[25,36-38]. Interestingly, recent data suggests that under stress CSE can translocate to mitochondria, and H_2S produced by mitochondrially located CSE can be used as a substrate for the production of ATP[39]. This might be an additional protective mechanism in the setting of ischemia/hypoxia.

The association between the mRNA expression of CSE and the outcome after human renal transplantation is an indication that CSE could have protective effects in human ischemia/reperfusion injury similar to those we have found in murine models. The observation that CBS is not differentially expressed during the transplant process, nor is associated with outcome after transplantation, indicates a central role for CSE in human renal tissue. We found an increase in mRNA expression of CSE shortly after reperfusion in humans, which was similar to the results from rats. The decrease in CSE expression found at the later time points after reperfusion in the murine model could not be confirmed in the human study due to the absence of biopsies late after reperfusion. The question remains whether this early decrease is associated with

a functional mechanism where CSE is downregulated or inhibited during renal repair and regeneration, or the inflammatory response to injury.

Taken together, our data reveal a key role for CSE in the regulation of oxidative stress induced following renal ischemia, most likely through the production of H_2S. The modulation of CSE expression and activity may be a therapeutic target in settings of hypoxia.

SUPPLEMENTARY FIGURE



Supplementary Figure 1. Representative photomicrographs showing structural damage caused by ischemia/reperfusion injury in wildtype and $CSE^{-/-}$ animals. Original magnification 200x.

CONCISE MATERIALS AND METHODS

Collection of human material

Human renal transplant biopsies were obtained from brain-dead or living donors (n = 50) at three different time points: just before donation (before start of preservation), at the end of cold ischemia and approximately 45 min after reperfusion in the recipient. Control kidneys were taken from the unaffected part of kidneys from patients undergoing nephrectomy for renal cell carcinoma (n=6).

Animals

CSE deficient mice ($CSE^{-/-}$) were generated using conventional techniques as described before[3]. Wildtype C57BL/6J littermates (WT) were used as control animals. Male Wistar rats (250–300 g) were used for time-course experiments.

IRI protocol

Renal ischemia/reperfusion in mice was performed as described previously16. In short, both renal pedicles were clamped for 30 minutes using non-traumatic vascular clamps through a midline abdominal incision under general anesthesia (ketamine/xylazine). Core body temperature was maintained at 37°C in all groups using heat pads and lamps. NaHS injection (1 mg/kg) was given intraperitoneally 15 minutes before clamping. Mice were terminated after 1 day of reperfusion and samples were collected.

Cell culture experiments

To generate CSE overexpressing cells, human embryonic kidney 293 (HEK293) cells were transfected with the pIRES2-EGFP (mock) or pIRES2-EGFP-CSE vector in 24- or 96-well plates. For induction of ROS, Antimycin A (50 µg/mL) with or without NaHS (1-1000 µM) was given for 30 min. Subsequently, cells were incubated with 15 µM dyhidroethidine or 5 µM MitoSOX for 15 minutes.

Renin measurement

Renin immunohistochemistry was performed as described before[41]. Subsequently, the amount of glomeruli with renin-positive juxtaglomerular cells were counted. In a second analysis, the amount of renin positive cells per glomerulus were counted. For both measurements, the complete cortex was analyzed in a blinded fashion.

TissueFAXS analysis

CSE-Alexa 488 / CD31-TRITC / DAPI triple stained sections were scanned using a fluorescent microscope fitted with an automated acquisition system (TissueFAXS, TissueGnostics GmbH, Vienna, Austria). Percentages of CSE+, CD31+ and CSE+CD31+ cells were measured using TissueQuest software (TissueGnostics), using an algorithm based on the recognition of nuclei and their associated cytoplasm. Ki67 and γH2AX stained sections were quantified in this manner as well.

Statistical analysis

Data were analyzed using Mann-Whitney U tests, One-Way ANOVA or Kruskall Wallis tests where appropriate. Bonferroni or Dunns postcorrection was applied where multiple comparisons

were made. Normality was tested using the Kolmogorov–Smirno test. A value of $p<0.05$ was considered statistically significant. Data are expressed as the mean ± SEM (Standard Error of the Mean) unless otherwise indicated.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude towards Sippie Huitema and Marian Bulthuis for their excellent technical and logistical support, and Shetuan Zhang from the Department of Physiology, Queen's University, Kingston, Ontario, Canada for donating the pIRES2-EGFP plasmid.

This study was financially supported by a Kolff Grant from the Dutch Kidney Foundation to EMB, a grant from the Groningen University Institute for Drug Exploration (GUIDE) to EMB, a ZonMW Medium Investment Grant to JLH and an operating grant from Canadian Institutes of Health Research to RW.

None of the authors report a conflict of interest, financial or otherwise, regarding this paper.

REFERENCES

1. Wang R: Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? FASEB J 16: 1792–1798, 2002
2. Szabó C: Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov 6: 917–935, 2007
3. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R: H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 322: 587–590, 2008
4. Mustafa AK, Sikka G, Gazi SK, Steppan J, Jung SM, Bhunia AK, Barodka VM, Gazi FK, Barrow RK, Wang R, Amzel LM, Berkowitz DE, Snyder SH: Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. Circ. Res. 109: 1259–1268, 2011
5. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC: Hypertension in mice lacking the gene for endothelial nitric oxide synthase. Nature 377: 239–242, 1995
6. Sen U, Munjal C, Qipshidze N, Abe O, Gargoum R, Tyagi SC: Hydrogen sulfide regulates homocysteine-mediated glomerulosclerosis. Am. J. Nephrol. 31: 442–455, 2010
7. Yang G, Wu L, Bryan S, Khaper N, Mani S, Wang R: Cystathionine gamma-lyase deficiency and overproliferation of smooth muscle cells. Cardiovascular Research 86: 487–495, 2010
8. Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R, Szabó C: Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proc Natl Acad Sci USA 106: 21972–21977, 2009
9. Zanardo RCO, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL: Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J 20: 2118–2120, 2006
10. Miller TW, Wang EA, Gould S, Stein EV, Kaur S, Lim L, Amarnath S, Fowler DH, Roberts DD: Hydrogen sulfide is an endogenous potentiator of T cell activation. Journal of Biological Chemistry 287: 4211–4221, 2012
11. Mustafa AK, Gadalla MM, Sen N, Kim S, Mu W, Gazi SK, Barrow RK, Yang G, Wang R, Snyder SH: H_2S signals through protein S-sulfhydration. Sci Signal 2: ra72, 2009
12. Krishnan N, Fu C, Pappin DJ, Tonks NK: H_2S -Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. Sci Signal 4: ra86, 2011
13. Sen N, Paul BD, Gadalla MM, Mustafa AK, Sen T, Xu R, Kim S, Snyder SH: Hydrogen sulfide-linked sulfhydration of NF-κB mediates its antiapoptotic actions. Mol. Cell 45: 13–24, 2012
14. Blackstone E, Morrison M, Roth MB: H_2S induces a suspended animation-like state in mice. Science 308: 518, 2005
15. Khan AA, Schuler MM, Prior MG, Yong S, Coppock RW, Florence LZ, Lillie LE: Effects of hydrogen sulfide exposure on lung mitochondrial respiratory chain enzymes in rats. Toxicol. Appl. Pharmacol. 103: 482–490, 1990
16. Bos EM, Leuvenink HGD, Snijder PM, Kloosterhuis NJ, Hillebrands J-L, Leemans JC, Florquin S, van Goor H: Hydrogen sulfide-induced hypometabolism prevents renal ischemia/reperfusion injury. J Am Soc Nephrol 20: 1901–1905, 2009
17. Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L, Jiao X, Scalia R, Kiss L, Szabó C, Kimura H, Chow CW, Lefer DJ: Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci USA 104: 15560–15565, 2007
18. Minamishima S, Bougaki M, Sips PY, Yu JD, Minamishima YA, Elrod JW, Lefer DJ, Bloch KD, Ichinose F: Hydrogen sulfide improves survival after cardiac arrest and cardiopulmonary resuscitation via a nitric oxide synthase 3-dependent mechanism in mice. Circulation 120: 888–896, 2009
19. Jha S, Calvert JW, Duranski MR, Ramachandran A, Lefer DJ: Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. Am J Physiol Heart Circ Physiol 295: H801–6, 2008
20. Bos EM, Snijder PM, Jekel H, Weij M, Leemans JC, van Dijk MCF, Hillebrands J-L, Lisman T, van Goor H, Leuvenink HGD: Beneficial effects of gaseous hydrogen sulfide in hepatic ischemia/reperfusion injury. Transpl Int 25: 897–908, 2012
21. Kimura Y, Kimura H: Hydrogen sulfide protects neurons from oxidative stress. FASEB J 18: 1165–1167, 2004
22. Kimura Y, Goto Y-I, Kimura H: Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. Antioxid Redox Signal 12: 1–13, 2010
23. Li Z, Yang J, Huang H: Oxidative stress induces H2AX phosphorylation in human spermatozoa. FEBS Lett. 580: 6161–6168, 2006

24. Tripatara P, Patel NSA, Brancaleone V, Renshaw D, Rocha J, Sepodes B, Mota-Filipe H, Perretti M, Thiemermann C: Characterisation of cystathionine gamma-lyase/hydrogen sulphide pathway in ischaemia/reperfusion injury of the mouse kidney: An in vivo study. Eur. J. Pharmacol. 606: 205–209, 2009
25. Tripatara P, Patel NSA, Collino M, Gallicchio M, Kieswich J, Castiglia S, Benetti E, Stewart KN, Brown PAJ, Yaqoob MM, Fantozzi R, Thiemermann C: Generation of endogenous hydrogen sulfide by cystathionine gamma-lyase limits renal ischemia/reperfusion injury and dysfunction. Lab. Invest. 88: 1038–1048, 2008
26. Blackstone E, Roth MB: Suspended Animation-Like State Protects Mice from Lethal Hypoxia. Shock 27: 370–372, 2007
27. Morrison ML, Blackwood JE, Lockett SL, Iwata A, Winn RK, Roth MB: Surviving blood loss using hydrogen sulfide. J Trauma 65: 183–188, 2008
28. House JD, Brosnan ME, Brosnan JT: Characterization of homocysteine metabolism in the rat kidney.Biochem J 328 (Pt1): 287–292, 1998
29. Wiseman H, Halliwell B: Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J 313 (Pt 1): 17–29, 1996
30. Cadet J, Delatour T, Douki T, Gasparutto D, Pouget JP, Ravanat JL, Sauvaigo S: Hydroxyl radicals and DNA base damage. Mutat Res 424: 9–21, 1999
31. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM: DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 273: 5858–5868, 1998
32. Lu M, Liu Y-H, Goh HS, Wang JJX, Yong Q-C, Wang R, Bian J-S: Hydrogen sulfide inhibits plasma renin activity. J Am Soc Nephrol 21: 993–1002, 2010
33. Whitfield NL, Kreimier EL, Verdial FC, Skovgaard N, Olson KR: Reappraisal of H_2S / sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. Am J Physiol Regul Integr Comp Physiol 294: R1930–7, 2008
34. Oh G-S, Pae H-O, Lee B-S, Kim B-N, Kim J-M, Kim H-R, Jeon SB, Jeon WK, Chae H-J, Chung H-T: Hydrogen sulfide inhibits nitric oxide production and nuclear factor-kappaB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radic Biol Med 41: 106–119, 2006
35. Calvert JW, Coetzee WA, Lefer DJ: Novel insights into hydrogen sulfide-mediated cytoprotection. Antioxid Redox Signal 12: 1203–1217, 2010
36. Fu Z, Liu X, Geng B, Fang L-P, Tang C-S: Hydrogen sulfide protects rat lung from ischemia-reperfusion injury. Life Sci. 82: 1196–1202, 2008
37. Tyagi N, Moshal KS, Sen U, Vacek TP, Kumar M, Hughes WM, Kundu S, Tyagi SC: H_2S protects against methionine-induced oxidative stress in brain endothelial cells. Antioxid Redox Signal 11: 25–33, 2009
38. Wang Q, Liu H-R, Mu Q, Rose P, Zhu Y-Z: S-propargyl-cysteine protects both adult rat hearts and neonatal cardiomyocytes from ischemia/hypoxia injury: the contribution of the hydrogen sulfide-mediated pathway. J. Cardiovasc. Pharmacol. 54: 139–146, 2009
39. Fu M, Zhang W, Wu L, Yang G, Li H, Wang R: Hydrogen sulfide (H_2S) metabolism in mitochondria and its regulatory role in energy production. Proc Natl Acad Sci USA 109: 2943–2948, 2012
40. Zhao W, Zhang J, Lu Y, Wang R: The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J. 20: 6008–6016, 2001
41. Fraune C, Lange S, Krebs C, Hölzel A, Baucke J, Divac N, Schwedhelm E, Streichert T, Velden J, Garrelds I, Danser AHJ, Frenay A-R, van Goor H, Jankowski V, Stahl RA, Nguyen G, Wenzel UO: AT1 antagonism and renin inhibition in mice: pivotal role of targeting angiotensin II in chronic kidney disease. Am J Physiol Renal Physiol 2012

FULL MATERIALS AND METHODS

Collection of human renal transplant biopsies

Cortical kidney needle biopsy specimens were taken from donors (n=50) during procurement, pre-implantation, and ~45 min after reperfusion. Biopsy specimens were taken using a 16-gauge needle (Acecut, TSK Laboratory, Japan). Biopsies were fixed in 4% formalin for paraffin embedding or snap frozen in liquid nitrogen and stored at –80°C for mRNA isolation. Some donor kidneys were obtained outside our medical center, which impeded the collection of some biopsies at the procurement time point. Some kidneys were retrieved at our hospital, but allocated outside our medical center, obstructing the collection of some pre-implantation and reperfusion biopsies. Subjects were included when the following criteria were fulfilled: donor and recipient age between 18 and 70 years; cold ischemic time less than 25 hours; anastomosis time less than 60 min; no reclamping after completing the anastomosis; and first kidney recipients.

Collection of control renal tissue

Control human renal tissue used for analysis of normal CSE expression patterns was taken from the unaffected part of kidneys removed for the treatment of renal cell carcinoma (n=6).

RNA isolation and real-time PCR analysis

A nucleosin II RNA isolation kit (Macherey-Nagel GmbH, Germany) was used. RNA was eluted in 20 µl of RNase-free water. RNA concentration was measured using a Nanodrop spectrophotometer. RNA quality was confirmed by agarose gel electrophoresis. For cDNA synthesis, 1 µg of RNA was used and reverse transcription was performed with a Quantitect Reverse Transcription kit (Qiagen GmbH, Germany). Gene expression assays –were bought from Applied Biosystems. (Foster City, Ca, USA). For human PCR; CBS: Hs00163925_m1, CSE: Hs00542284_m1. HPRT was custom made, with sequence: F: 5'-GGCAGTATAATCCAAAGATG GTCAA-3'; R: 5'-GTCTGGCTTATATCCAACACTTCGT-3'. For rat material; CBS: Rn00560948_m1, CSE: Rn00567128_m1. HPRT was custom made, with sequence: F: 5'-GCCCTTGACTATAATGA GCACTTCA-3'; R: 5'-TCTTTTAGGCTTTGTACTTGGCTTTT-3'. Changes in CSE, CBS and HPRT mRNA were confirmed by two-step quantitative RT-PCR on an AB Prism 7900HT Sequence detector (Applied Biosystems). The PCR profile consisted of 15 min at 95°C, followed by 40 cycles with heating of 95°C for 15 s and cooling to 60°C for 1 min. Data were analysed with SDS2.3 (Applied Biosystems). All results were normalized to the expression of HPRT in the sample.

Immunofluorescence

Immunofluorescent labeling was performed as described previously. Briefly, Cryosections (4 µm) were dried and acetone fixed. Primary antibodies were applied for 1 hour, and thereafter incubated with appropriate secondary antibodies for 30 minutes. Slides were mounted in Aqua/ polymount (Polysciences Inc., Warrington, PA, USA) containing DAPI (1.5 µg/ml). The following primary antibodies were used: mouse monoclonal CSE (kindly donated by dr. N. Nishi, Kagawa Medical School, Japan). Mouse monoclonal CD31 (Dako), Rabbit polyclonal Ki67 (Abcam), and mouse monoclonal γH2AX (Millipore). Secondary antibodies include Alexa Fluor 488 rabbit anti-

mouse IgG2a (Invitrogen), TRITC-conjugated rabbit anti mouse IgG1 (Southern Biotech), Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen), Cy3-conjugated goat anti-rabbit IgG (Invitrogen)

TissueFAXS analysis

CSE-Alexa 488 / CD31-TRITC / DAPI triple stained sections were scanned using a fluorescent microscope (Zeiss AxioObserver.Z1, Carl Zeiss AG, Oberkochen, Germany) fitted with an automated acquisition system (TissueFAXS, TissueGnostics GmbH, Vienna, Austria). Non-representative images of the section were removed (e.g. folds, edges). Percentages of CSE+, CD31+ and CSE+CD31+ cells were measured using TissueQuest software (TissueGnostics), using an algorithm based on the recognition of nuclei and their associated cytoplasm. In human renal transplant biopsies, intensity of the fluorescence signal was measured in the cytoplasm using the TissueQuest software. In addition, Ki67 and γH2AX stained sections were quantified in this manner. Random backgating analysis was performed in each sample, where negativity, positivity, and double positivity of cells in the scatterplots was confirmed by manual investigation of the associated photomicrograph.

Ly-6G and renin immunohistochemistry

Immunohistochemical staining for Ly-6G/C positive granulocytes was performed as described previously[1]. In short, paraffin embedded sections were stained for Ly-6G using rat-anti-mouse Ly6G/C-FITC IgG2b antibody, followed by rabbit-anti-FITC and HRP-conjugated goat-anti-rabbit antibodies. Full slide images were captured at 40x magnification using a Hamamatsu NanoZoomer 2.0HT, and images were analysed using HistoQuest software (TissueGnostics). For renin, we used a polyclonal antibody as described before[2,3].

Measurement of necrosis

Formalin fixed, paraffin embedded coronal renal sections were stained using the Periodic Acid Schiff (PAS) method using standard procedures. Whole slides were digitized using an Aperio ScanScope digital slide scanner (Aperio Technologies inc, Vista, Ca, USA) at 20x magnification and the accompanying Aperio Imagescope software was used to measure the necrotic renal cortical area relative to the total renal cortical area.

Plasma biochemical analysis

Creatinine measurements were performed by our hospital clinical chemistry research services using conventional methods.

Renin measurement

Subsequently, the amount of glomeruli with renin-positive juxtaglomerular cells were counted. In a second analysis, the amount of renin positive cells per glomerulus were counted. For both measurements, the complete cortex was scanned in a blinded fashion.

Animals

For renal IRI and H_2S production rate measurement studies, CSE deficient mice ($CSE^{-/-}$) were generated using conventional techniques as described before[4]. Wildtype C57BL/6J littermates

(WT) were used as control animals. Time-course expression of CSE mRNA after IRI was examined in male Wistar rats (250–300 g; Harlan, Zeist, the Netherlands, n=6 per group).

IRI protocol

In mice, both renal pedicles of WT or CSE $^{-/-}$ animals (n=9-14 per group) were clamped for 30 minutes using non-traumatic vascular clamps through a midline abdominal incision under general anesthesia (100 mg/kg ketamine, 8 mg/kg xylazine). After removing the clamps, kidneys were inspected for restoration of blood flow and the muscle and skin layers were sutured with 5-0 vicryl stitches (Ethicon, New Jersey, USA). Body temperature was maintained at 37°C using heat pads and lamps. Sham-operated animals received the exact same procedure, without placement of the clamps on the renal pedicles. Treatment with NaHS (1 mg/kg, freshly dissolved in sterile phosphate buffered saline (PBS)) was given 15 minutes before application of the clamps through an intraperitoneal injection. Control animals received an injection of PBS at that time. Mice were sacrificed 24 hours after reperfusion.

In the Wistar rat model, a unilateral approach was used. Animals were anesthetized with 2% isoflurane with a flow of 0.6 l/min of 100% oxygen and a left-flank incision was made. Renal vessels of the left kidney were dissected and clamped with non-traumatic vascular clamps for 45 min. After removal of the clips, renal reperfusion was confirmed visually and muscle and skin layers were sutured using 4-0 vicryl stitches (Ethicon, New Jersey, USA). Reperfusion times were 30 min, 90 min, 6 h, 1 day, 4 days, 9 days, 14 days or 21 days (n=5-6 per group).

Cell culture

Human embryonic kidney 293 (HEK293) cells were used for *in vitro* experiments, cultured under standard conditions using Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 10% fetal bovine serum. All experiments were independently reproduced at least three times.

Transfection

For experiments utilizing overexpression of CSE, HEK293 cells were transfected with the pIRES2-EGFP (mock) or pIRES2-EGFP-CSE vector. For this purpose, cells were seeded in 24- or 96-well plates and grown until subconfluent. Subsequently, transfection was performed according to manufacturers' instructions using Lipofectamine 2000 reagent (Invitrogen). Transfection efficiency and time course of expression was determined using fluorescence microscopy for EGFP. For experiments investigating oxidative stress and the protein expression of CSE, cells were used or harvested 72 hours after transfection.

Cell viability measurement

Alamar Blue reagent (Invitrogen) was used according to manufacturers' protocol to measure cellular viability in the first 96 hours after transfection.

H_2S production rate measurement

Whole kidneys were taken from untreated CSE $^{-/-}$ or WT mice and snap frozen in liquid nitrogen. For measurement of H_2S production, renal tissue was homogenized and measurement was performed as described previously[5].

H_2S measurement in medium

Supernatants of control, pIRES2-EGFP and pIRES2-EGFP-CSE transfected cells was collected 72 hours after transfection and H_2S measurement was performed as described previously using the methylene blue assay[6].

Western Blot – rat tissue

Total protein lysates in RIPA buffer (Thermo Scientific, MA, USA) were supplemented with protease inhibitor cocktail (Sigma–Aldrich, MA, USA). Protein concentrations were determined using the BioRad DC protein assay (Bio-Rad, VA, USA), according to manufacturer's protocol. Equal amounts of protein were loaded onto 12% SDS-polyacrylamide gels. Aspecific binding of the antibodies was prevented using 5% milk in Tris-buffered saline-0.1% Tween-20 (TBST). Then membranes were incubated with the primary antibody solution in 5% milk in TBST, overnight at 4°C (for CSE: Proteintech rabbit polyclonal CSE antibody 12217-1-AP (1:1000), for Ywhaz (which was used as a housekeeping protein): Abcam anti 14-3-3 zeta rabbit polyclonal antibody ab51129 (1:500)). Afterwards membranes were incubated with secondary antibody (goat anti-rabbit IgG horseradish peroxidase) 1:500 in 5% milk in TBST. All antibody incubations were followed by washing with TBST. Membranes were immediately developed by luminol-chemiluminescence according to manufacturer's instructions, and digital images taken with the Bio-Rad ChemiDoc MP system quantified using Bio-Rad Image Lab 4.01.

Western Blot – cultured cells

Cultured HEK293 cells were obtained 72 hours after transfection and lysed in lysis buffer (0.5 M EDTA, 1 M Tris-Cl, pH 7.4, 0.3 M sucrose, 1 µg/ml antipain hydrochloride, 1 mM benzamidine hydrochloride hydrate, 1 µg/ml leupeptin hemisulfate, 1 mM 1,10-phenanthroline monohydrate, µ1 M pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide). Equal amounts of protein were boiled and electrophoretically separated by SDS-PAGE and transferred to nitrocellulose membrane as described previously[7]. The dilutions of primary antibody were 1:5000 for α-Tubulin and 1:1000 for CSE. Horseradish peroxidase-conjugated antibody was used as secondary antibody. The immunoreactions were visualized by ECL and exposed to X-ray film (Kodak Scientific Imaging film).

Model of Antimycin A induced oxidative stress and measurement of reactive oxygen species

HEK293 cells were cultured in 96-well plates until subconfluent and starved in serum-free medium for 24 hours. Antimycin A (50 µg/mL) with or without NaHS (1-1000 µM) was given for 30 min to induce intracellular production of ROS. Subsequently, cells were washed two times with PBS to remove Antimycin and NaHS and incubated with 15 µM dyhidroethidine (DHE, Invitrogen) or 5 µM MitoSOX (Invitrogen) for 15 minutes and washed three times with PBS. Images were directly made using an inverted fluorescent microscope and analyzed for intensity of fluorescence.

Statistical analysis

Data were analyzed using GraphPad PRISM 5.0 (GraphPad, San Diego, USA) or SPSS 16.0 (SPSS inc., Chicago, IL, USA) using Mann-Whitney U tests, One-Way ANOVA or Kruskall Wallis tests where

appropriate. Bonferroni or Dunns postcorrection was applied where multiple comparisons were made. Normality was tested using the Kolmogorov–Smirno test. Association between mRNA and protein levels and GFR were analysed using linear regression. For histopathological scoring, both kidneys of each animal were analysed and a mixed-effects model was used taking the animal of origin into account. A value of $p<0.05$ was considered statistically significant. Data are expressed as the mean ± SEM (Standard Error of the Mean) unless otherwise indicated.

Approval

For clinical studies, all procedures and use of anonymized human tissue were performed according to recent national guidelines, and were approved by our institutional review board. For the animal studies, experimental procedures were in agreement with institutional and legislator regulations and approved by the local committee for animal experiments.

REFERENCES FOR FULL MATERIALS AND METHODS

1. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJD, Kirschning CJ, Akira S, van der Poll T, Weening JJ, Florquin S: Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest 115: 2894–2903, 2005
2. Krebs C, Hamming I, Sadaghiani S, Steinmetz OM, Meyer-Schwesinger C, Fehr S, Stahl RAK, Garrelds IM, Danser AHJ, van Goor H, Contrepas A, Nguyen G, Wenzel U: Antihypertensive therapy upregulates renin and (pro)renin receptor in the clipped kidney of Goldblatt hypertensive rats. Kidney Int 72: 725–730, 2007
3. Fraune C, Lange S, Krebs C, Hölzel A, Baucke J, Divac N, Schwedhelm E, Streichert T, Velden J, Garrelds I, Danser AHJ, Frenay A-R, van Goor H, Jankowski V, Stahl RA, Nguyen G, Wenzel UO: AT1 antagonism and renin inhibition in mice: pivotal role of targeting angiotensin II in chronic kidney disease. Am J Physiol Renal Physiol 2012
4. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R: H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 322: 587–590, 2008
5. Zhao W, Zhang J, Lu Y, Wang R: The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J. 20: 6008–6016, 2001
6. Yang G, Wu L, Wang R: Pro-apoptotic effect of endogenous H_2S on human aorta smooth muscle cells. FASEB J 20: 553–555, 2006
7. Yang G, Sun X, Wang R: Hydrogen sulfide-induced apoptosis of human aorta smooth muscle cells via the activation of mitogen-activated protein kinases and caspase-3. FASEB J 18: 1782–1784, 2004

6

HYDROGEN SULFIDE-PRODUCING ENZYMES IN PREGNANCY AND PREECLAMPSIA

KM Holwerda
EM Bos
A Rajakumar
C Ris-Stalpers
MG van Pampus
A Timmer
JJHM Erwich
MM Faas
H van Goor
AT Lely

Published in Placenta

Reference: Placenta, 2012. Jun;33(6): 518–521
Digital object identifier (DOI): 10.1016/j.placenta.2012.02.014

ABSTRACT

Preeclampsia, a human pregnancy specific disorder, is characterized by an anti-angiogenic state. As hydrogen sulfide (H_2S) has pro-angiogenic and anti-oxidative characteristics, we hypothesized that H_2S levels could play a role in the pathogenesis of preeclampsia and studied the placental expression of the H_2S-producing enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS). CBS and CSE protein are expressed in the fetal-placental endothelium and CBS only in Hofbauer-cells. CBS mRNA expression is decreased ($p=0.002$) in early-onset preeclampsia, while CSE mRNA is unchanged. Thus, down regulation of CBS during early onset preeclampsia may result in less H_2S-production and may aid in the anti-angiogenic state.

INTRODUCTION

Preeclampsia (PE), a human pregnancy specific disorder, is characterized by placental ischemia and maternal endothelial dysfunction[1]. The poorly perfused and ischemic placenta releases excess amounts of anti-angiogenic factors causing generalized endothelial damage[2,3].

Hydrogen sulfide (H_2S) is produced from the amino acid L-cysteine by two pyridoxal 5' phosphate-dependent enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS). H_2S induces vasorelaxation by opening ATP-sensitive K-channels in smooth muscle cells and up regulates vascular endothelial growth factor[4,5]. Furthermore, H_2S also has antioxidant capacity by direct scavenging of nitrogen or reactive oxygen species[6]. CBS and CSE are down regulated in several cardiovascular and pulmonary diseases[7]. Exogenous H_2S (NaHS) administration is proposed as a novel therapy in animal models of cardiovascular and ischemic diseases[7,8]. CBS is also an important enzyme in the homocysteine pathway, since homocysteine is converted to cystathionine by CBS. Pregnant CBS transgenic mice show a moderate increase of homocysteine which associated with blunted endothelial-dependent relaxation in arteries[9].

We hypothesized that H_2S, because of its pro-angiogenic and anti-oxidative characteristics and the involvement of CBS in homocysteine degradation, might play a role in the pathogenesis of PE. The aim of the present study was to identify and compare the expression and localization of CBS and CSE in placental tissue from both normotensive and early- and late-onset PE.

MATERIALS AND METHODS

Collections of samples

Placental biopsies were obtained from patients (n=36) with early-onset PE, late-onset PE and mode of delivery matched healthy pregnant controls after informed consent. The local UMCG Medical Ethical Committee approved the study. PE was defined according to the standards of the International Society for the Study of Hypertension in Pregnancy: diastolic blood pressure of > 90 mm Hg and proteinuria ≥ 300 mg/24 hours[10]. PE present before 34 weeks of gestation was defined as early-onset, these patients delivered by Cesarean section. Intra-uterine growth restriction (IUGR) was defined as birth weight under the tenth percentile.

As previously described, placental cryosections were stained[11] with mouse monoclonal antibodies against CSE (1:100, donated by dr. N. Nishi, Kagawa Medical School, Japan) and CBS (1:250, Abnova, Tapei, Taiwan). Primary antibody was replaced by PBS in negative controls. For immunofluorescence double staining, CD31 (1:100, Sigma-Aldrich, St. Louis, MO) was used.

For real time RT-PCR, RNA was isolated from several parts of the placenta, pooled, and purified as previously described[12]. We analyzed mRNA expression of CBS and CSE using Assay-on-Demand Gene Expression (Applied Biosystems, USA). PSMD4 (proteasome non-ATPase regulatory subunit 4) was used as a housekeeping gene, the expression of this gene was constant over the four study groups[13].

Total protein from placental biopsies was extracted and Western blotting analysis was performed according to published procedures[14,15]. CBS and CSE monoclonal antibodies (Abnova) were used at 1:500 dilution.

Table 1 - Clinical characteristics of pregnant women with early- and late-onset preeclampsia and mode of delivery matched controls.

	Delivery by Cesarean section		Spontaneous delivery	
	Control pregnancy	Early-onset preeclampsia	Control pregnancy	Late-onset preeclampsia
Number	9	10	8	9
Age (years)	33 (26 – 35)	31 (25 – 26)	27 (27 – 37)	30 (26 – 33)
Gestational age at delivery (weeks+days)	38+6 (38+6 – 39+3)	28+6 (27+5 – 30+3)**	39+3 (38+5 – 41+0)	38+1 (37+0 – 39+3)¥
Systolic blood pressure (mmHg)	132 (125 – 147)	178 (166 – 190)*	120 (118 – 120)	155 (150 – 163)¥¥
Diastolic blood pressure (mmHg)	80 (80 – 85)	110 (110 – 115)**	76 (70 – 80)	97 (92 – 105)¥¥
Proteinuria (grams/24 hours)	0	3.0 (0.9 – 4.9)**	0	0.8 (0.7 – 0.8)¥¥
Birth weight (grams)	3880 (3610 – 4085)	933 (713 – 1103)**	3390 (3180 – 3700)	2770 (2569 – 3071)¥
HELLP syndrome	0	0	0	0
IUGR	0	2 (20)	0	1 (11)

Data are expressed as median (interquartile range) and numbers (%).
For statistical analysis Mann-Whitney U test and Fisher exact test were used.
*p<0.05, **p<0.001, when compared to healthy pregnancy with delivery by Cesarean section.
¥p<0.05, ¥¥p<0.001, when compared to healthy pregnancy with spontaneously delivery.

RESULTS AND DISCUSSION

The major finding of this study is that CBS mRNA expression is significantly down regulated in placental villous tissue derived from pregnancies complicated by early-onset PE when comparing to mode of delivery matched controls (Figure 1a). Furthermore, we demonstrated that CBS and CSE are mainly localized in the endothelium in the fetal vessels from the chorionic- and stem-villi (Figure 2a). The endothelial origin of both enzymes is confirmed by double staining with CD31 (Figure 2b). Hofbauer cells express CBS (Figure 2a). There were no differences in CBS/CSE in protein expression between PE and delivery matched controls (Figure 1b). However, CBS/CSE protein expression was significantly down regulated in all placentae after spontaneous delivery compared to Cesarean delivery (Figure 1b).

Although protein expression of CBS and CSE was not affected by PE, we found a down regulation of mRNA of CBS in early-onset PE. This discrepancy between mRNA and protein expression is remarkable, but has been reported previously[16]. In ischemic brain tissue, decrease in mRNA corresponded to decreased CBS-activity and H_2S-production, while protein levels did not[16]. We did not evaluate CSE - and CBS-activity or H_2S-production. However, Patel et al. showed that H_2S is endogenously produced in the placenta, production rate was increased under low-oxygen levels[17]. Another study confirmed placental catalytic CBS-activity by converting homocysteine[18]. So far, in PE no CBS- and CSE-activity or H_2S-production is reported. Therefore, the exact role of endogenous H_2S in PE needs to be elucidated.

Differential CBS mRNA expression may be gestation related, as has been previously documented for other genes, however no differences in CBS mRNA expression measured in first-trimester and term human placentae were reported[19]. For late-onset PE, placental CBS mRNA expression is not altered compared to healthy pregnancies (Figure 1a). This is in line with the growing evidence that there are differences between the pathophysiology of early- and late-onset PE[20]. In our total study group, 3 patients with a pregnancy complicated by IUGR were present. The data of these patients with respect to mRNA and protein expression fitted well within their study groups.

CBS and CSE protein, but not mRNA expression was significantly down regulated in control and PE placentae after spontaneous delivery compared to Cesarean delivery. This is in line with a recent report of down regulation of the enzymes and reduced H_2S-production in the myometrium during labor[21]. Increased turnover of the enzymes could be involved in transition of the labor process.

The fetal endothelial expression of CBS and CSE is in accord with the expressions in other organs[22]. Moreover, we showed expression of CBS by Hofbauer cells. Hofbauer cells or fetal tissue macrophages are placental immune cells; several studies suggest that Hofbauer cells play a direct role in placental vasculogenesis[23]. Although CSE has been shown to be expressed by macrophages[24], the expression of CBS by macrophages has not been reported. The expression of CBS by Hofbauer cells may be in line with the role of these cells in vasculogenesis.

In conclusion, the present study provides novel insights into the expression of H_2S-producing enzymes during normal and PE. Future studies will compare the placental CBS/CSE-activity and H_2S-production in pregnancies and explore the possible therapeutic role of H_2S during PE.

Figure 1

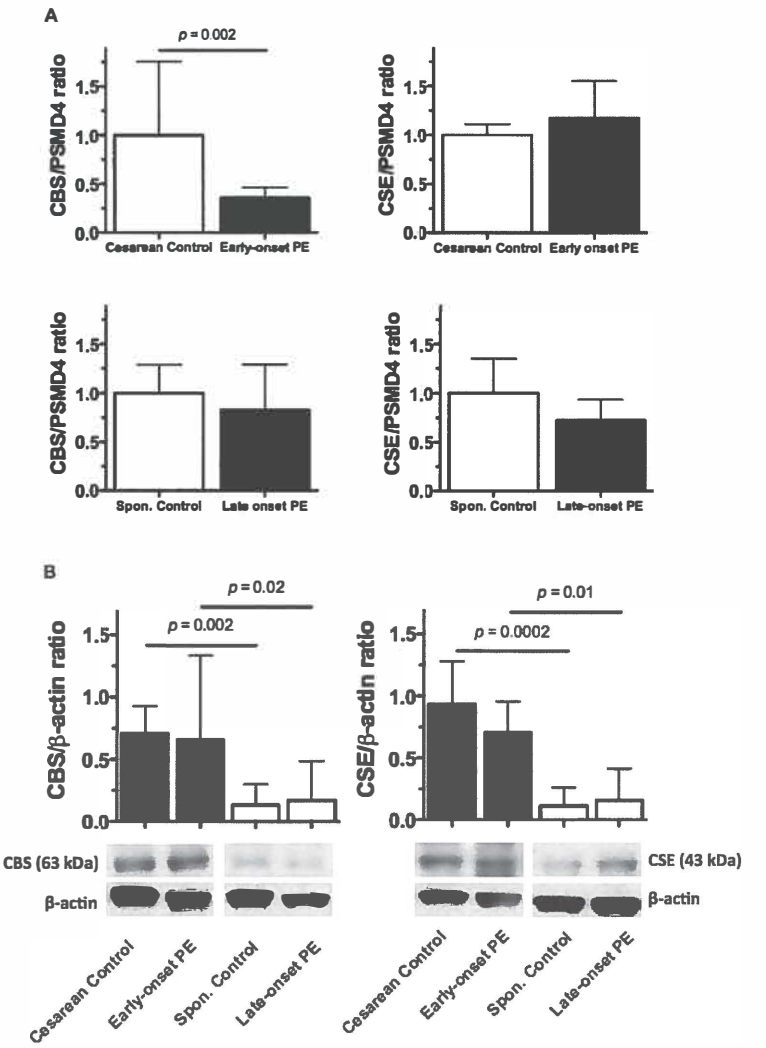


Figure 1 - Placental expression of CBS and CSE mRNA and protein in healthy pregnancy and pregnancy complicated by early- and late-onset preeclampsia.
(A) CBS mRNA expression is down regulated in the early-onset preeclampsia group, compared to the mode for delivery matched group (delivery by Cesarean section). No differences were observed in CBS mRNA expression in the late-onset PE group or in CSE mRNA expression in all four groups. There was no difference in mRNA expression between the late- and early-onset PE groups of both enzymes. (B) Quantitative Western blot analysis showed a significant down regulation of both CBS and CSE in all spontaneous delivery placentae, compared to the Cesarean groups. No differences were observed in expression of both enzymes between both PE groups and mode of delivery matched controls. Bands 1 – 4 show representative Western blots for CBS in placentae derived from respectively Cesarean controls, early-onset PE, spontaneous controls and late-onset PE. Bands 5 – 8 show representative Western blots for CSE in the same groups. For all figures, median and interquartile range is given. For statistical analysis, Mann-Whitney U test was used. Quantification of protein expression was performed by measuring band intensity using NIH ImageJ software.

Figure 2

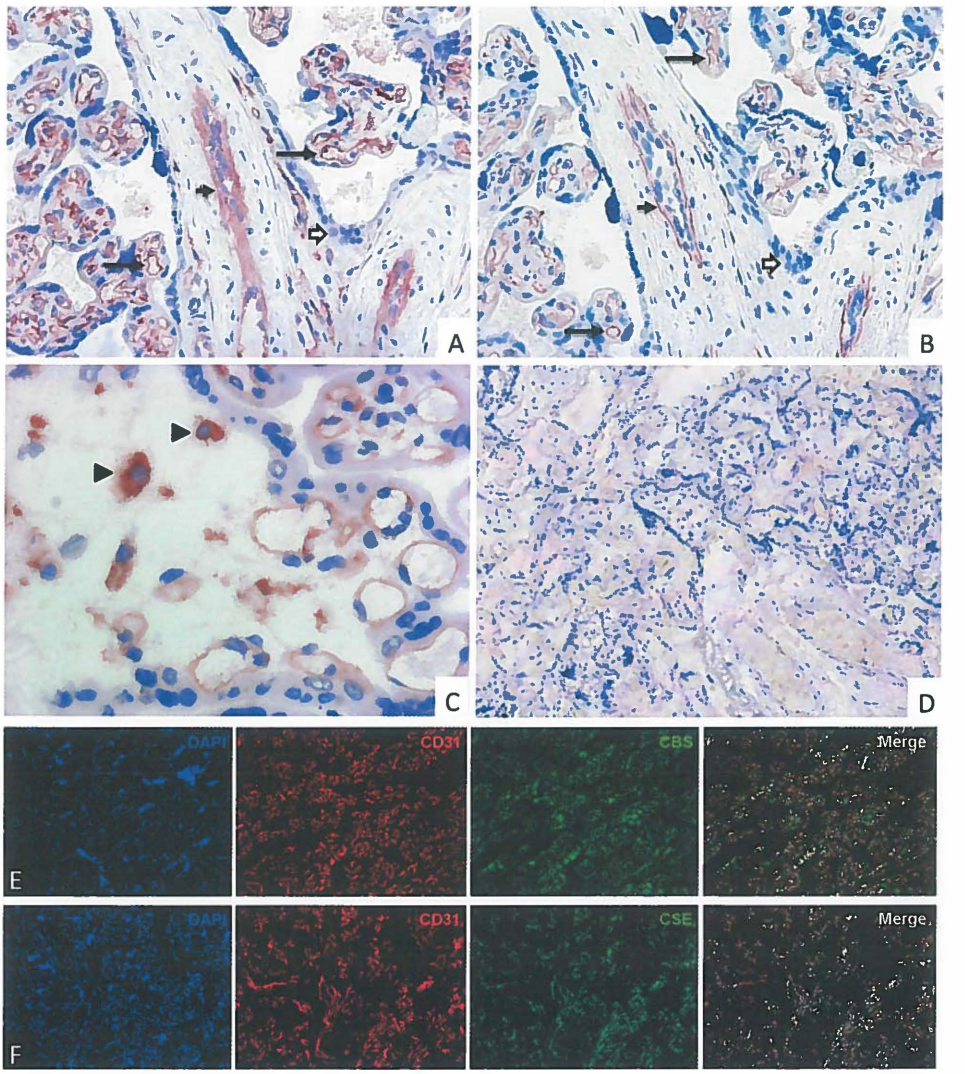


Figure 2 - Placental localization of CBS and CSE protein expression in healthy pregnancy IHC and IF staining was performed on all samples of the 4 groups (n=36).

No differences in localization were observed between groups; therefore only representative cryostat sections of control placental villous tissue stained for CBS (A, C, F) and CSE (B, E) are shown. Positive staining is shown in fetal endothelial cells in chorionic villi (long arrow), fetal endothelial cells in stem villi (closed short arrow) and in Hofbauer cells (arrow-head). Syncytio- and cytotrophoblasts are negative (open short arrow). No positive staining is observed in the negative control, in which the primary antibody was replaced by PBS (D).

For the IF staining (E, F), nuclear staining (DAPI) is shown in blue, CD31 protein is shown in red, CSE and CBS protein in green, and co-localization of CD31 with CBS or CSE in white. IF staining confirmed the endothelial expression of both CSE and CBS protein. All images were taken at the same exposure and with a magnification of x20 (A, B, D, E, F) or x40 (C).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Dutch Kidney Foundation (KBSO 11.014), a Mandema Stipendium (to dr. A.T. Lely) from the University Medical Center Groningen, and a grant from the Groningen University Institute of Drug Exploration (GUIDE). The authors greatly acknowledge the technical contribution of Sippie Huitema and Theo Borghuis, and the PREHAT-team for processing placental tissues.

REFERENCES

1. James JL, Whitley GS, Cartwright JE,. Pre-eclampsia: fitting together the placental, immune and cardiovascular pieces. J.Pathol. 2010; 221:363-378.
2. Sibai B, Dekker G, Kupferminc M,. Pre-eclampsia. Lancet 2005; 365:785-799.
3. Redman CW, Sargent IL,. Latest advances in understanding preeclampsia. Science 2005; 308:1592-1594.
4. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA,. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J.Clin.Invest. 2003; 111:649-658.
5. Gilbert JS, Ryan MJ, LaMarca BB, Sedeek M, Murphy SR, Granger JP,. Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. Am.J.Physiol.Heart Circ.Physiol. 2008; 294:H541-50.
6. Young BC, Levine RJ, Karumanchi SA,. Pathogenesis of preeclampsia. Annu.Rev. Pathol. 2010; 5:173-192.
7. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA, CPEP Study Group,. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N.Engl.J.Med. 2006; 355:992-1005.
8. Maynard SE, Venkatesha S, Thadhani R, Karumanchi SA,. Soluble Fms-like tyrosine kinase 1 and endothelial dysfunction in the pathogenesis of preeclampsia. Pediatr.Res. 2005; 57:1R-7R.
9. Geelhoed JJ, Fraser A, Tilling K, Benfield L, Davey Smith G, Sattar N, Nelson SM, Lawlor DA,. Preeclampsia and gestational hypertension are associated with childhood blood pressure independently of family adiposity measures: the Avon Longitudinal Study of Parents and Children. Circulation 2010; 122:1192-1199.
10. Jayet PY, Rimoldi SF, Stuber T, Salmon CS, Hutter D, Rexhaj E, Thalmann S, Schwab M, Turini P, Sartori-Cucchia C, Nicod P, Villena M, Allemann Y, Scherrer U, Sartori C,. Pulmonary and systemic vascular dysfunction in young offspring of mothers with preeclampsia. Circulation 2010; 122:488-494.
11. Wang R,. Hydrogen sulfide: a new EDRF. Kidney Int. 2009; 76:700-704.
12. Wang R,. Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter?. FASEB J. 2002; 16:1792-1798.
13. Wagner CA,. Hydrogen sulfide: a new gaseous signal molecule and blood pressure regulator. J.Nephrol. 2009; 22:173-176.
14. Abe K, Kimura H,. The possible role of hydrogen sulfide as an endogenous neuromodulator. J.Neurosci. 1996; 16:1066-1071.
15. Siebert N, Cantre D, Eipel C, Vollmar B,. H_2S contributes to the hepatic arterial buffer response and mediates vasorelaxation of the hepatic artery via activation of K(ATP) channels. Am.J.Physiol.Gastrointest. Liver Physiol. 2008; 295:G1266-73.
16. Wang MJ, Cai WJ, Zhu YC,. Mechanisms of angiogenesis: role of hydrogen sulphide. Clin. Exp.Pharmacol.Physiol. 2010; 37:764-771.
17. Szabó C,. Hydrogen sulphide and its therapeutic potential. Nat.Rev.Drug Discov. 2007; 6:917-935.
18. Predmore BL, Lefer DJ,. Development of hydrogen sulfide-based therapeutics for cardiovascular disease. J.Cardiovasc.Transl. Res. 2010; 3:487-498.
19. Bos EM, Leuvenink HG, Snijder PM, Kloosterhuis NJ, Hillebrands JL, Leemans JC, Florquin S, van Goor H,. Hydrogen sulfide-induced hypometabolism prevents renal ischemia/reperfusion injury. J.Am.Soc. Nephrol. 2009; 20:1901-1905.
20. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM,. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). Hypertens. Pregnancy 2001; 20:IX-XIV.
21. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R,. H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 2008; 322:587-590.
22. Zhu XY, Liu SJ, Liu YJ, Wang S, Ni X,. Glucocorticoids suppress cystathionine gamma-lyase expression and H_2S production in lipopolysaccharide-treated macrophages. Cell Mol.Life Sci. 2010; 67:1119-1132.

23. Tang Z, Abrahams VM, Mor G, Guller S,. Placental Hofbauer cells and complications of pregnancy. Ann.N.Y.Acad.Sci. 2011; 1221:103-108.

24. Seval Y, Korgun ET, Demir R,. Hofbauer cells in early human placenta: possible implications in vasculogenesis and angiogenesis. Placenta 2007; 28:841-845.

7

PERSPECTIVES ON DONOR ORGAN PRECONDITIONING IN HEART BEATING RENAL TRANSPLANTATION

EM Bos
HGD Leuvenink
H van Goor
RJ Ploeg

Adapted from a manuscript published in Kidney International

Reference: Kidney Int., 2007. Oct;72(7):797-805
Digital Object Identifier (DOI): 10.1038/sj.ki.5002400

ABSTRACT

Major improvements in immunosuppressive treatment, surgical techniques, and treatment of post-transplant complications have contributed considerably to improved outcome in renal transplantation over the past decades. Yet, these accomplishments have not led to similar improvements in transplant outcome when the results of living and deceased donors are compared. The enormous demand for donor kidneys has allowed for the increase in acceptance of suboptimal donors. The use of brain dead patients as organ donors has had a tremendous positive influence on the number of renal transplants. Unfortunately, the physiologically abnormal state of brain death has a negative effect on transplant outcome. The fact that transplanted kidneys derived from brain dead donors have a decreased viability indicates that potential grafts are already damaged before retrieval and preservation. In this review, we present an overview of the current knowledge of (patho)-physiological effects of brain death and its relevance for renal transplant outcome. In addition, several options for therapeutic intervention during brain death in the donor with the goal to improve organ viability and transplant outcome are discussed.

INFERIOR SURVIVAL OF DECEASED DONOR KIDNEYS AFTER TRANSPLANTATION

Transplant outcome achieved with kidneys from living donors is far superior when compared to grafts obtained from deceased donors[1;2] (Table 1). The persistent donor organ shortage has caused longer waiting lists and an increasing percentage of patients that die while waiting. As a consequence, a gradual shift towards accepting suboptimal donors has taken place. The use of older and more marginal donors is now routine and the number of non-heart beating donors has increased significantly over the past years[1;3]. Twenty years ago, the typical donor was under the age of thirty, fairly healthy and died of traumatic cerebral injury. Today, the average donor is over fifty years old and the main cause of death is intracranial hemorrhage. The improvements that were made in treatment regimen of the recipient, organ preservation, reduction of cold ischemia time, and better allocation of donor organs have been masked by the use of lower quality donors. In the past, much effort was directed towards post-transplantation immunosuppression and preservation of organs during transport. Now, risk factors and conditions prior to organ retrieval in the donor need to be recognized for their impact on donor organ viability. The detrimental effects of brain death on renal transplant outcome[4;5] have been convincingly shown in the experimental setting. In clinical studies, though, it is difficult to reveal that brain death itself has an independent influence on transplant outcome, since living and deceased donors differ on more aspects than just the death of the brain. However, when survival rates are stratified for age, grafts from deceased donors have worse survival within each age-group – even in the relatively young group of 25-36 year old donors[2].

In 2003, 14.853 renal transplantations were performed in the United States. Of those, 8.389 kidneys originated from deceased donors and 6.464 were retrieved from living donors[1]. Yet, at the end of 2003, 57.211 patients were on the waiting list to receive a renal transplant. For a patient enlisted in 2000, the median time to transplant was more than three years. Unfortunately, not every patient survived long enough to receive a transplant, which resulted in approximately 3 000 deaths on the waiting list in that same year. The increased demand for donor kidneys has provoked

Table 1 - 1- and 5-year graft survival for living and deceased donors following renal or liver transplantation.

	1-Year Survival		5-Year Survival	
Organ transplanted	**2001-2002**	**2002-2003**	**1997-2002**	**1998-2003**
Kidney				
Living donor	94.3%	94.6%	78.6%	79.2%
Deceased donor	88.7%	89.0%	65.7%	66.2%
Liver				
Living donor	79.3%	80.1%	78.1%	71.2%
Deceased donor	80.6%	81.4%	64.1%	65.4%

Source: UNOS/OPTN.

a large shift towards living kidney donation in the U.S.[1] In fact, the number of living kidney donors in the U.S. surpassed the number of deceased donors in 2000[1;6]. Still, the total number of kidneys obtained from living donors is lower since in living donation only one kidney can be donated, while in deceased donors both kidneys are retrieved. In many European countries the use of living donors has increased as well over the past decade, albeit more modestly[3].

In their 1995 landmark paper, Terasaki et al.[2] showed that graft survival for living unrelated donation (LURD) is superior compared to deceased donation, even though the average HLA-haplotype matching is worse in LURD. Long-term outcome after LURD is similar to that of parental or offspring donors[7]. This indicates that poor survival of cadaveric grafts cannot be solely attributed to differences in immunogenicity. Graft performance is affected by many other factors. Donor variables such as age, gender, race, terminal serum creatinine, history of hypertension and cause of death all affect transplantation outcome[8-10]. Deceased donors tend to be older than living donors[1], however within each age category, survival rates of living donor grafts are significantly higher than those of deceased donor grafts[2]. Preservation and cold ischemia time (CIT) influence transplant outcome, and for logistical reasons, CIT is longer on average in deceased donor transplantation. For renal transplantation, CIT of less than 24 hours does not influence outcome after renal transplantation[2;11;12].

Despite the fact that grafts obtained from deceased donors have inferior outcome, these transplants have prevented death for many people on dialysis. Deceased donor kidney recipients have a 68% reduced risk of mortality compared to similar patients who stay on the waiting list receiving dialysis treatment[13]. The shortage of donor organs culminated in the use of extended criteria donation (ECD). ECD includes brain dead donors who are older than 60 years, or are aged over 50 years in combination with at least two of the following risk factors: a history of hypertension, a terminal serum creatinine >1.5 mg/dL or a cerebrovascular cause of death. The number of ECD-derived kidneys has seen marginal but steady increase over the past years[1], even though long term allograft survival of ECD-derived kidneys is inferior compared to non-ECD kidneys[1;14] (Figure 1). Initial doubt about the advantages of this type of donor has not been sustained. Relative mortality risk analysis has shown that the short-term risk of death in ECD kidney recipients is more than five times higher when compared to standard therapy with dialysis while waiting for a non-ECD kidney. At 226 days after transplantation, however, the risk becomes equal and is lower thereafter. In addition, the long term cumulative mortality is significantly lower in ECD kidney recipients[15]. Kidneys discarded by transplant centers twice or more for reasons of poor organ quality showed worse initial non-function and long term renal performance. Five year graft and patient survival, however, were not significantly different from control kidneys that were immediately accepted[16]. Direction of ECD kidneys towards those patients with a high risk of mortality on the waiting list could therefore be beneficial. On the other hand, caution is advised, since allocating compromised kidneys towards the most vulnerable patients could have its own negative effects on outcome. Progressively increasing waiting lists and subsequent prolonged waiting time in the years to come will further extend the benefit of ECD transplantation. When ECD kidneys are optimally allocated, they can be a valuable source to reduce the wide gap between the number of donor organs needed and

those that actually become available. Counteracting the deleterious effects that the state of brain death has on these organs could increase the benefit even further.

BRAIN DEATH, SYSTEMIC CHANGES AND CLINICAL COURSE

The beating heart has always been considered the classic sign of life. An unconfirmed story about the famous 16th century anatomist Andreas Vesalius reports about an incident that happened at a public dissection on a Spanish nobleman during the times of the Inquisition. On opening the thorax, Vesalius found, to the shock of the crowd, that the heart was still beating. Vesalius was taken to court by the deceased man's family, appalled about this crime to their relative, who must have been alive as his heart was still beating. Myth or not, it illustrates that the presence of a heartbeat was synonymous with life in those days.

After the report by Mollaret and Goulon in 1959[17] which described comatose patients with vital functions sustained by mechanical ventilation, the definition of death became a major point of discussion. Due to improved techniques, the heart was kept functioning in these patients while mechanical ventilation oxygenated the blood. Judged by their appearance, patients did not look deceased, however, it was clear that normal, self-sustained function would never be regained. In 1963, before a consensus had been reached on the implications of this irreversible coma, the first kidney was retrieved from a brain dead, heart-beating donor and transplanted by the Belgian surgeon Alexandre[18]. In an effort to overcome problems that led to controversy in obtaining organs from deceased donors, but also to decrease the burden on the relatives of brain dead patients on life support, an ad hoc committee of the Harvard Medical School proposed to add irreversible coma to the death criterion in 1968[19]. This report generated considerable medico-legal discussion and resulted in most countries adopting a concept of death that originates from this proposition. The definition of brain death that the committee proposed concerned the following mandatory criteria: (1) unawareness of and unresponsiveness to external stimuli, (2) no spontaneous movements or breathing, (3) absent reflexes and (4) a flat electroencephalogram (EEG). Caution is required for conditions that can elicit similar symptoms, such as hypothermia, hypotension or intoxication, and therefore these have to be ruled out.

Brain death as such is the terminal phase of a sequence of events frequently commencing with cerebral trauma or cerebrovascular hemorrhage. When the patient is declared brain dead, this chain of events has already affected the organs. Systemic and hormonal changes arise immediately when intracranial pressure increases. Hence, brain death is not the stationary condition as perceived from the outside, but a dynamic and rather unphysiological course of events that influences a number of (patho-)physiological processes in the human body.

Hemodynamic changes

Following cerebral trauma or injury, the primary rise in intracranial pressure causes additional damage to the cerebrum, which triggers parasympathetic activity and results in a decreased systemic blood pressure. The continued rise in intracranial pressure leads to herniation of the brain stem through the foramen magnum, which is accompanied by arterial compression and ultimately occlusion with progressive ischemic damage. When the pontine part of the brain

Figure 1

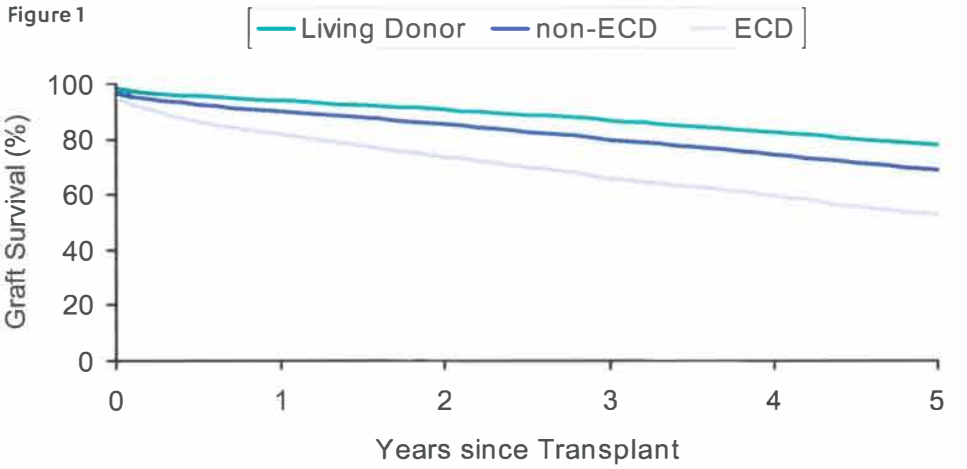


Figure 1 – Graft survival over a period of 5 years for living-, non-ECD- and ECD derived renal grafts. Source: UNOS/OPTN.

stem becomes ischemic, sympathetic stimulation, together with the persisting parasympathetic activity, will cause the Cushing reflex, which was already described in 1902 by the American neurosurgeon Harvey Cushing[20]. The Cushing reflex consists of multiple disturbances in the physiology of cerebrally injured patients, including bradycardia, hypertension and an irregular breathing pattern. Ultimately, when the entire brain stem has become ischemic, the vagal cardiomotor nucleus is affected and solitary sympathetic stimulation will occur. As a result, massive catecholamine release, systemically as well as from myocardial sympathetic nerve endings, cause an increase in heart rate and leads to vasoconstriction with increased vascular resistance and blood pressure[21-23]. This process is referred to as the sympathetic- or catecholamine storm and is considered to be an attempt of the body to raise arterial blood pressure above the elevated intracranial pressure as an ultimate effort to restore perfusion of the cerebrum.

The rise in serum epinephrine levels has been reported to be as high as 100-1,000 fold higher compared to normal values in animal models of brain death[23-26]. The magnitude of catecholamine release is related to the severity of brain damage. The faster the rise in intracranial pressure, the higher the peak in catecholamine levels[23]. Also, serum norepinephrine and dopamine concentrations are vastly increased after onset of brain death. The values of catecholamine release in animal experiments appear to be similar to those described in the clinical situation[27]. In addition, a parallel cardiac response to brain injury is seen, as demonstrated by even higher levels of myocardial catecholamines compared to the serum[28] leading to injury of myocytes[21;29]. The catecholamine induced increase in vascular resistance can be severe, reaching four times higher levels than basal values in the rat kidney[26]. This causes renal blood flow to decrease by a factor of 2.4 and supports the hypothesis that the rigorous decline in organ perfusion leads to ischemic damage of potential grafts.

Over time, sympathetic pathways are deactivated due to ischemia of the spinal cord. This leads to a gradual decrease of the hyperdynamic state with a subsequent decline in blood

pressure, heart rate and cardiac output to normal or subnormal values. Ultimately, a state of hypoperfusion is reached, which is harmful to the potential donor kidneys. Prolonged brain death results in high rates of tubular necrosis[30]. Many brain dead patients need hemodynamic support during this phase, and receive vasopressors and/or anti-diuretic hormone (ADH).

Hormonal changes

In addition to the catecholamines, other, hormonal alterations take place. There is evidence that some residual cerebral blood flow and hypothalamic function can persist after brain death[31-33]. In most brain dead patients, however, a gradual decrease in the release of adrenocorticotropic hormone (ACTH) and ADH is seen[24;25;34], which is associated with cardiovascular failure that eventually causes the requirement of hemodynamic support. The failure to keep ADH levels in the range needed for a normal osmolarity[24;25;31] has been suggested as the cause of diabetes insipidus in up to 78% of patients[34;35]. A more recent explanation is the downregulation of aquaporin-2 (AQP-2) channels[36] which could affect water re-uptake in the renal collecting ducts. Free circulating triiodothyronine (T3) gradually decreases after brain death[34;35;37-40], but not every study has found comparable results concerning the serum concentrations of T3 and T3-related hormones, such as T4 and TSH[32;39-41].

Any acute stress will enhance the condition known as "diabetes of injury", consisting mainly of hyperglycemia caused by increased gluconeogenesis and insulin resistance. Intensive insulin therapy is now applied in many intensive care units (ICU) and as a result mortality in surgical ICU's has been greatly reduced when strict glycemic control is achieved[42]. A recent study has indicated that in the medical ICU, overall mortality was only decreased after intensive insulin therapy in patients who stayed in the ICU for three or more days[43]. Morbidity, however, was reduced among all patients. A lower incidence of newly acquired renal injury, earlier weaning from mechanical ventilation, and a quicker discharge from the ICU and from the hospital were observed[43]. The rise in serum creatinine was attenuated by the maintenance of normoglycemia. Also, evidence now exists that insulin therapy reduces the inflammatory response. ICAM-1 and C-reactive protein are both decreased in the serum of ICU patients receiving intensive insulin treatment[44;45]. Thus, the use of intensive insulin therapy in brain dead patients could therefore attenuate renal damage, reduce inflammation and enhance donor organ viability resulting in a better transplantation outcome.

EXPERIMENTAL BRAIN DEATH MODELS

To obtain a better insight in the (patho-)physiological processes that occur during brain death, standardized models are necessary to explain the discrepancies in outcome between deceased and living donor transplantation. In transplant studies, the use of a brain death model is important, since the deceased donor is still the major source of donor organs. The induction of injury to the central nervous system under controlled conditions closes the gap between experimental transplant models and daily clinical reality. Valid experimental models have been developed in which the detrimental effects of brain death can be studied and possible interventions evaluated[46-49]. Various research groups have studied brain death in the rat model. Epidural

hematoma is simulated using an inflatable catheter inserted through a trepanation in the skull. Inflation of the catheter causes cerebral damage followed by edema, a rise in intracranial pressure and eventually herniation of the brain stem. The models vary in details such as the velocity of balloon catheter inflation and the use of hemodynamic support. Brain death can be confirmed – as in the human situation – by the absence of the apnea reflex, cornea reflex and observation of the typical course of blood pressure changes. The effects of brain death induction in the current animal models of brain death closely mirror observations from the clinical situation.

In recent years, the use of animal models with brain death has made it clear that organ quality is significantly affected and frequently diminished in brain dead animals. In contrast to the clinical circumstances, in the animal model, heterogeneity is reduced and the pathophysiology of cerebral injury leading to brain death in donors can be studied in far greater detail.

PATHOPHYSIOLOGICAL EFFECTS OF BRAIN DEATH ON RENAL FUNCTION AND STRUCTURES

Before 1997, the concept of brain death did not exist in Japan. Patients who would be considered brain dead and eligible for organ donation in the U.S. or Europe were kept in a coma until cardiac arrest. This presented Nagareda et al.[30] with the unique opportunity to investigate the time course of the effects of brain death on the kidney up to 48 days. Their study revealed that the mean urinary sodium output increased during the first 14 days, mean urine osmolarity was above normal on the first day but decreased gradually and urine volume during the first 14 days was high as a consequence of the cerebral injury related diabetes insipidus. On histological examination, degenerative changes of renal structures were found, including vacuolization, atrophy and necrosis of renal proximal and distal tubules. Advancing glomerulitis and progressing periglomerulitis expressed inflammatory changes. Periglomerular fibrosis and proliferation of the arterial intima and glomerular endothelium reflected the structural changes in the kidney.

In experimental conditions in rats, renal function is already negatively affected during four hours of brain death followed by inferior results after reperfusion in an isolated perfused kidney (IPK) set up. During IPK, urine volume and GFR were significantly higher than controls[50]. Interestingly, potassium excretion was increased in these kidneys, possibly explained by the depletion of ATP in these kidneys, which can trigger the opening of ATP-sensitive potassium channels (K_{ATP} channels). An impaired sodium/potassium homeostasis was observed after brain death in a renal slice model as well[51]. Organs can also become more prone to ischemia/reperfusion injury: livers derived from brain dead rats are more susceptible to cold storage-induced injury. This was demonstrated by a decreased survival after 20 hours of cold storage when compared to living donor livers stored equally long[52].

Renal tubular damage as a consequence of brain death can be observed in urine as well. Brush border enzymes, such as alkaline phosphatase (AP) and alanine amino peptidase (AAP), as well as the lysosomal enzyme N-acetyl-beta-D-glucosaminidase (NAG)[50], are released into the urine. Kidney injury molecule 1 (KIM-1), is a recently discovered brush border enzyme which is considered a marker of tubular damage, e.g. in ischemia/reperfusion injury[53]. As a result of

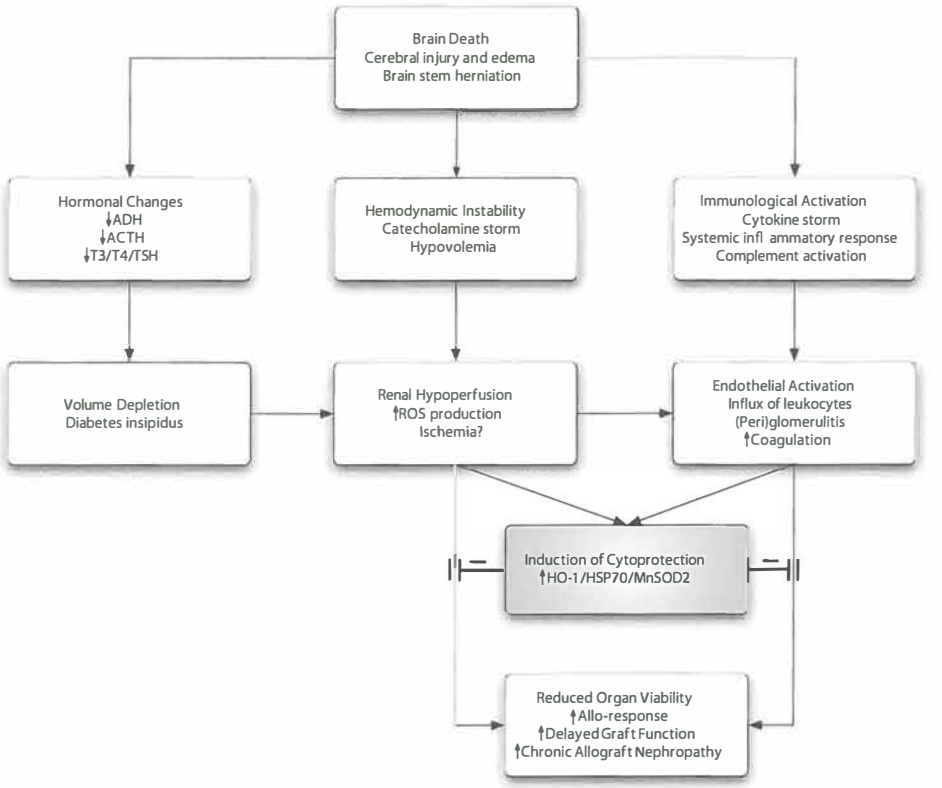


Figure 2 – Proposed model for the (patho)-physiological changes associated with brain death.

brain death, we found that KIM-1 is massively upregulated. Interestingly enough, it can be detected on the luminal side of the renal cortical tubule, but is also shed into the urine[54] which may simplify viability assessment of potential donor organs.

Immunological activation

In ischemia/reperfusion injury, a clear-cut correlation was found between endothelial injury and acute rejection. This association between the innate immune response and subsequent alloreactivity could be explained by Matzinger's danger hypothesis[55]. It is of importance that an increased immunogenicity is also observed in the brain dead donor organ as well. Endothelial activation is present with the upregulation of adhesion molecules (E-selectin, P-selectin, ICAM-1, VCAM-1) that promote the rolling, adhesion, diapedesis and subsequent leukocyte migration into the interstitium of the kidney[46;56-59]. Multiple cytokines and chemokines do play a role in the immunological response to cerebral injury. Upregulation of IL-1, IL-2, IL-6, TNF-α, TGF-β, IFN-γ, VEGF, MIP-1α, MIP-1β, MCP-1 and osteopontin have been reported[36;56;58,60]. The expression of the major histocompatibility complex (MHC) class II is increased as well[56]. Amplification of cytokines, chemokines and adhesion molecules causes a chemotactic gradient that promotes the influx of

leukocytes to the kidney. T-cells, macrophages and polymorphonuclear leukocytes are all found in higher quantities in donor kidneys during brain death[46;56;57;61].

After reperfusion, a large difference in neutrophil infiltration and P-selectin expression can be observed between living and cadaveric grafts. Koo et al.[62] showed that 53% of cadaveric renal allografts had increased neutrophil infiltration, against 0% of living related grafts. P-selectin expression was increased in 44% of cadaveric grafts, and 9% of living related grafts. In a syngeneic animal model of renal transplantation, short-term inflammatory changes to the kidneys were investigated by Kusaka et al[58]. The extent of leukocyte infiltration reaches its peak at 24 hours after transplantation in this syngeneic transplant model and corresponds with the levels of E- and P-selectin. After this period, the extent of immunological activation gradually decreases, but histological changes to the kidney can still be observed. Allotransplant experiments have shown that after experimental brain death, recipients of brain dead donor kidneys suffered from a greatly increased acute rejection rate[4]. Similar effects have been observed in other organs, such as lung[63] and heart[64]. When kidney allografts are treated with cyclosporine to prevent acute rejection, long-term renal function is adversely affected by brain death compared to syngeneic transplants. Thus, the state of brain death can also enhance the development of chronic renal transplant dysfunction[5].

Protection and Repair

Interestingly, not only detrimental or degenerative changes take place during brain death. Protective or recuperative mechanisms are induced as well. This is reflected by increased expression of the cytoprotective genes heme oxygenase-1 (HO-1), heat-shock protein 70 (HSP70) and manganese superoxide dismutase (MnSOD2)[36;61;65]. Kunzendorf et al.[66] showed that a prolonged duration of brain death positively influences long-term graft survival. The mechanism behind this observation could well be the delayed induction of protection or initiation of repair. In another study, increased HO-1 expression at organ retrieval was correlated with outcome after renal transplantation in the living donor setting[61]. Expression of HO-1 was not related to graft survival in deceased donor kidneys. Donor HO-1 gene polymorphisms have been associated with transplantation outcome[67]. Surprisingly, in a liver transplant study, livers with an initial low HO-1 expression prior to transplantation, but a high HO-1 expression after reperfusion, had superior outcome when compared to livers with high HO-1 expression at organ harvest[68]. These observations indicate that the ability to induce HO-1 is important, and not a high expression of HO-1 per se. Two different mechanisms should be considered here: while the increase in expression of HO-1 in living donors may initiate protection against the hits to the kidney during transplantation and thereafter, in deceased donors, on the other hand, HO-1 may well be a reflection of the level of stress to the kidney due to brain death.

EXPERIMENTAL AND CLINICAL INTERVENTIONS

Different concepts and approaches have been considered to counteract the detrimental effects caused by brain death (Table 2). Someresearch groups focus on the induction of protective proteins, while others aim at reducing the immune response. In a case-control study performed by Schnuelle

et al.[69], treatment of the donor with the vasopressors dopamine or noradrenalin was identified as an independent beneficial factor in renal transplant outcome. They confirmed these results in a study using the Eurotransplant database[70] and also in a recent study, where donor dopamine was found to be associated with a more rapid decrease in serum creatinine and better long-term survival[71]. Experimentally, dopamine treatment has shown beneficial effects in a model of brain death induced renal damage. Dopamine treatment resulted in HO-1 induction as well as an inhibition of P-selectin expression and decreased mononuclear infiltration[48]. In other experiments, the effects of ischemia/reperfusion and cold preservation were attenuated by low-dose dopamine treatment[72;73]. In vitro, decreased production of chemokines GRO-α, ENA78 and IL-8 in proximal tubular epithelial cells (PTEC) has been observed after dopamine treatment[74]. In endothelial cells, production of GROα and ENA78 was reduced, but IL-8 increased. In addition, dopamine pretreatment delayed expression of ICAM-1 and VCAM-1 after TNFα stimulation in these cells. These immunological effects seen in experimental conditions could be part of the explanation for the improved renal transplantation outcome after catecholamine treatment of the brain dead donor.

Selective upregulation of HO-1 has proven to be beneficial in different models of stress or damage, including ischemia/reperfusion[75] and experimental renal transplantation[76]. Due to its antioxidative, antiapoptotic and immune regulatory effects, HO-1 has become an extensively investigated protein in the search for protection against insults during and prior to the transplant

Table 2 - Overview of studies that investigated the effects of specific interventions on brain death related damage.

Study	Treatment	Main outcome
Human studies		
Kuecuek (2005)[79]	Steroids	Reduced expression of proinflammatory cytokines
Schnuelle (1999)[69]	Dopamine	Improved graft survival, less acute rejection
	Norepinephrine	Improved graft survival, less acute rejection
Schnuelle (2001)[70]	Catecholamines§	Improved graft survival
Schnuelle (2004)[71]	Dopamine	Improved graft survival, improved short-term renal function
Animal studies		
Coleman (2006)[83]	CEPO	Reduced expression of proinflammatory factors
Gasser (2002)[80]	rPSGL-Ig	Improved graft survival, reduced chronic rejection
Kotsch (2006)[77]	CoPP	Improved graft survival, reduced leukocyte infiltration
Pratschke (2001)[78]	rPSGL-Ig	Improved graft survival, reduced acute rejection
	Steroids	Improved graft survival, reduced acute rejection
Schaub (2004)[48]	Dopamine	Reduced monocyte infiltration, reduced expression of proinflammatory factors

§Catecholamine treatment was defined as administration of any of the following adrenergic substances: dopamine, norepinephrine, epinephrine, dobutamine..
CEPO, carbamylated erythropoietin; rPSGL-Ig, recombinant P-selectin glycoprotein ligand; CoPP, cobalt protoporphyrin (inducer of HO-1).

process. Experimental transplantation after upregulation of HO-1 by CoPP treatment in the brain dead donor resulted in improved renal allograft survival[77]. Also, a reduction was seen in the infiltration of $ED1^+$ monocytes/macrophages, $CD4^+$ T-cells and $CD8^+$ T-cells in the CoPP treated group. The application of novel and existing approaches in the field of HO-1 induction can therefore be regarded as a promising possibility to improve clinical renal transplant outcome.

Inhibition of the immunological activation due to brain death is one of the strategies to improve transplant outcome in experimental models. Glucocorticosteroid treatment of brain dead rats improved graft survival after transplantation to a level comparable with living donor transplants[78]. Steroids suppress cellular infiltration and expression of cytokines and the intensity of morphological changes are noticeably different in the recipients of a graft from an untreated brain dead donor. The only prospectively designed human study known to us has reported some promising results in reducing the expression of proinflammatory cytokines using steroid therapy[79]. Long term results after kidney transplantation, however, have yet to be reported.

Table 3 - Interventions that can counteract the negative effects of brain death on the kidney, or could be used for this purpose in the future.

Potential interventions for use in the brain dead organ donor
Hemodynamic
Catecholamines (Dopamine, Epinephrine, Norepinephrine)
Antidiuretic hormone (ADH)
Anti-inflammatory
Immunosuppressants (Glucocorticoids, Calcineurin inhibitors)
Monoclonal antibodies against cytokines (TNF-α, IFN-γ, IL-2, IL-6)
Inhibitors of chemokines (MCP-1, MIP-1α, MIP-1β)
Carbamylated recombinant human Erythropoietin (CEPO)
Recombinant P-Selectin Glycoprotein Ligand – Ig (rPSGL-Ig)
Induction of cytoprotection
HO-1 induction (Cobalt Protoporphyrin (CoPP))
HSP induction (Pyrrolidine Dithiocarbamate (PDTC), Geranylgeranylacetone (GGA))
Signal transduction
Selective inhibitors of kinases (JNK, p38, ERK, RhoA)
Gaseous substances
Hydrogen Sulfide (H_2S)
Carbon Monoxide (CO)
Nitrous Oxide (NO)
Modulation of gasotransmitter production
Hormonal
Intensive insulin therapy

Treatment of the donor with the recombinant soluble P-selectin glycoprotein ligand (rPSGL-Ig), an inhibitor of P- and E- and L-selectin, has also been shown as advantageous in experimental models[78,80]. Three days after transplantation, untreated brain dead donor kidneys showed severe tubular necrosis and mononuclear infiltration, whereas recipients where the donor was treated with rPSGL-Ig showed similar serum creatinine levels to living donor iso- and allograft recipients. In addition, rPSGL-Ig treatment was able to affect chronic transplant dysfunction in animals that received brain dead donor kidneys, reducing long-term graft injury to a level seen in the living donor situation.

Carbamylated recombinant human erythropoietin (CEPO) is an EPO derivative that does not have hematopoietic effects, but has tissue protective capacities in different models of neural damage such as stroke[81]. Recently, we found that CEPO can reduce the renal inflammatory response and attenuate the increase in serum creatinine levels due to brain death[82,83]. Also, an improved GFR was observed in kidneys derived from CEPO treated brain dead rats compared to untreated controls. The combination of immunomodulation and tissue protection could be effective in reducing brain death related damage.

In recent years, hydrogen sulfide (H_2S) has come to the attention of researchers in the field of ischemia/reperfusion injury. Many protective properties of the gas have been discovered, including induction of a hypometabolic state resembling hibernation. This state has been used for protection of kidneys during ischemia/reperfusion injury. Also, deficiency of CSE, one of the H_2S producing enzymes in the body, causes increased damage after renal ischemia. Interestingly, the amount of CSE mRNA expression in the donor prior to ischemia is associated with short term renal function after transplantation. Modulation of H_2S producing proteins in the brain dead organ donor, or addition of gaseous H_2S to the ventilator gases before organ retrieval might prevent renal ischemic injury and improve outcome after transplantation. These results must be first confirmed in the human setting, however.

PERSPECTIVES ON DONOR MANAGEMENT AND PRETREATMENT

The deleterious effects of brain death on the donor kidney provoke pathophysiological changes that have a negative impact on the outcome after transplantation. Ischemia of the brain results in non-function of the central nervous system and is associated with pertinent hemodynamic instability, hormonal changes and diminished perfusion. This abnormal physiological state induces pro-inflammatory changes in the potential donor organs that negatively affect function and cause an increased chance of acute rejection. These compromising changes in the donor urge us to develop treatment regimens for application during brain death.

The use of pharmacological interventions to provide optimal conditions for the donor organ and prevent the decline of renal function will become an important part of the entire donation and transplantation process. Reducing hemodynamic instability is crucial to maintain normal perfusion of organs. The use of catecholamines for this purpose would benefit renal transplant outcome. Caution is needed, however, since interventions that can be of benefit to

one organ may be detrimental to another. This was demonstrated by Schnuelle et al. in their analysis of catecholamine use in the donor[70]. Although renal transplant survival was increased, liver transplant outcome was not improved and cardiac results appeared to be adversely influenced by catecholamine administration in the donor. A randomized prospective clinical trial is currently underway to assess the effects of donor pretreatment with dopamine.

The application of intensive insulin therapy for strict glycemic control will be beneficial to prevent damage to the organs during brain death. It is not clear yet if the effect will be large enough, however, to have consequences for graft viability and transplant outcome.

The use of immunomodulators such as steroids or rPSGL-Ig has shown some promising results in experimental models. Counteracting inflammatory changes in the deceased donor kidney improved function and survival after transplantation. In fact, steroid treatment is effective in modulating the immune response in human organ donors[79]. Since all organs exhibit inflammatory changes as a result of brain death, immunomodulating treatment has a high probability to be of benefit for all transplanted organs.

The induction of protective mechanisms, such as HO-1 upregulation, is an important development in donor pretreatment. Initiation of protective pathways can diminish brain death related damage and ischemia/reperfusion injury. The products created during heme degradation by HO-1 are involved in cytoprotective processes. In addition, immunomodulating effects of HO-1 could be of use in the improvement of deceased donor transplantation. Another option is the addition of gaseous substances to the breathing air of brain dead donors. Carbon monoxide (CO) has demonstrated a beneficial effect in modulating ischemia/reperfusion injury[84] and low dose inhalation of CO after experimental renal transplantation prevents the development of chronic allograft nephropathy[85]. In addition, the use of H_2S donors or upregulation of H_2S producing enzymes hold promise in this field.

To date, many challenging opportunities do exist to counteract the deleterious effects of brain death on the donor kidney. A better characterization and understanding of the mechanisms of injury and repair that play a role during massive cerebral injury and its effect on potential donor organs will lead to novel treatment options. As a result, the outcome after deceased donor organ transplantation may improve, and approach that of living donors.

ACKNOWLEDGEMENTS

The authors would like to thank Steven McGuire and Wynand B.W.H. Melenhorst for their critical review of this manuscript.

REFERENCES

1. U.S. Department of Health and Human Services. 2004 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 1994-2003. 2005.
2. Terasaki PI, Cecka JM, Gjertson DW et al. High survival rates of kidney transplants from spousal and living unrelated donors. N.Engl.J.Med. 333:333-336, 1995
3. 2004 Annual Report of the Eurotransplant International Foundation. 2005.
4. Pratschke J, Wilhelm MJ, Kusaka M et al. Accelerated rejection of renal allografts from brain-dead donors. Ann.Surg. 232:263-271, 2000
5. Pratschke J, Wilhelm MJ, Laskowski I et al. Influence of donor brain death on chronic rejection of renal transplants in rats. J.Am.Soc. Nephrol. 12:2474-2481, 2001
6. Davis CL, Delmonico FL. Living-donor kidney transplantation: a review of the current practices for the live donor. J.Am.Soc. Nephrol. 16:2098-2110, 2005
7. Futagawa Y, Waki K, Gjertson DW et al. Living-unrelated donors yield higher graft survival rates than parental donors. Transplantation 79:1169-1174, 2005
8. Pirsch JD, Ploeg RJ, Gange S et al. Determinants of graft survival after renal transplantation. Transplantation 61:1581-1586, 1996
9. Port FK, Bragg-Gresham JL, Metzger RA et al. Donor characteristics associated with reduced graft survival: an approach to expanding the pool of kidney donors. Transplantation 74:1281-1286, 2002
10. Ojo AO, Wolfe RA, Held PJ et al. Delayed graft function: risk factors and implications for renal allograft survival. Transplantation 63:968-974, 1997
11. Ploeg RJ, van Bockel JH, Langendijk PT et al. Effect of preservation solution on results of cadaveric kidney transplantation. The European Multicentre Study Group. Lancet 340:129-137, 1992
12. Koning OH, Ploeg RJ, van Bockel JH et al. Risk factors for delayed graft function in cadaveric kidney transplantation: a prospective study of renal function and graft survival after preservation with University of Wisconsin solution in multi-organ donors. European Multicenter Study Group. Transplantation 63:1620-1628, 1997
13. Wolfe RA, Ashby VB, Milford EL et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. N.Engl.J.Med. 341:1725-1730, 1999
14. Ojo AO, Hanson JA, Meier-Kriesche H et al. Survival in recipients of marginal cadaveric donor kidneys compared with other recipients and wait-listed transplant candidates. J.Am. Soc. Nephrol. 12:589-597, 2001
15. Merion RM, Ashby VB, Wolfe RA et al. Deceased-donor characteristics and the survival benefit of kidney transplantation. JAMA 294:2726-2733, 2005
16. Dahmane D, Audard V, Hiesse C et al. Retrospective follow-up of transplantation of kidneys from 'marginal' donors. Kidney Int. 69:546-552, 2006
17. Mollaret P, Goulon M. [The depassed coma (preliminary memoir).]. Rev.Neurol.(Paris) 101:3-15, 1959
18. Machado C. The first organ transplant from a brain-dead donor. Neurology 64:1938-1942, 2005
19. A definition of irreversible coma. Report of the Ad Hoc Committee of the Harvard Medical School to Examine the Definition of Brain Death. JAMA 205:337-340, 1968
20. Cushing H. Some experimental and clinical observations concerning states of increased intracranial tension. Am.J.Med.Sci 124:375-400, 1902
21. Novitzky D. Selection and management of cardiac allograft donors. Curr.Opin.Cardiol. 11:174-182, 1996
22. Novitzky D, Wicomb WN, Cooper DK et al. Prevention of myocardial injury during brain death by total cardiac sympathectomy in the Chacma baboon. Ann.Thorac.Surg. 41:520-524, 1986
23. Shivalkar B, van Loon J, Wieland W et al. Variable effects of explosive or gradual increase of intracranial pressure on myocardial structure and function. Circulation 87:230-239, 1993
24. Chen EP, Bittner HB, Kendall SW et al. Hormonal and hemodynamic changes in a validated animal model of brain death. Crit Care Med. 24:1352-1359, 1996
25. Sebening C, Hagl C, Szabo G et al. Cardiocirculatory effects of acutely increased intracranial pressure and subsequent brain death. Eur.J.Cardiothorac.Surg. 9:360-372, 1995

26. Herijgers P, Leunens V, Tjandra-Maga TB et al. Changes in organ perfusion after brain death in the rat and its relation to circulating catecholamines. Transplantation 62:330-335, 1996

27. Powner DJ, Hendrich A, Nyhuis A et al. Changes in serum catecholamine levels in patients who are brain dead. J.Heart Lung Transplant. 11:1046-1053, 1992

28. Mertes PM, Carteaux JP, Jaboin Y et al. Estimation of myocardial interstitial norepinephrine release after brain death using cardiac microdialysis. Transplantation 57:371-377, 1994

29. Biswas SS, Chen EP, Bittner HB et al. Brain death further promotes ischemic reperfusion injury of the rabbit myocardium. Ann.Thorac. Surg. 62:1808-1815, 1996

30. Nagareda T, Kinoshita Y, Tanaka A et al. Clinicopathology of kidneys from brain-dead patients treated with vasopressin and epinephrine. Kidney Int. 43:1363-1370, 1993

31. Arita K, Uozumi T, Oki S et al. The function of the hypothalamo-pituitary axis in brain dead patients. Acta Neurochir.(Wien.) 123:64-75, 1993

32. Sugimoto T, Sakano T, Kinoshita Y et al. Morphological and functional alterations of the hypothalamic-pituitary system in brain death with long-term bodily living. Acta Neurochir.(Wien.) 115:31-36, 1992

33. Petty GW, Mohr JP, Pedley TA et al. The role of transcranial Doppler in confirming brain death: sensitivity, specificity, and suggestions for performance and interpretation. Neurology 40:300-303, 1990

34. Gramm HJ, Meinhold H, Bickel U et al. Acute endocrine failure after brain death? Transplantation 54:851-857, 1992

35. Howlett TA, Keogh AM, Perry L et al. Anterior and posterior pituitary function in brain-stem-dead donors. A possible role for hormonal replacement therapy. Transplantation 47:828-834, 1989

36. Schuurs TA, Gerbens F, van der Hoeven JA et al. Distinct transcriptional changes in donor kidneys upon brain death induction in rats: insights in the processes of brain death. Am.J.Transplant. 4:1972-1981, 2004

37. Novitzky D, Cooper DK, Reichart B. Hemodynamic and metabolic responses to hormonal therapy in brain-dead potential organ donors. Transplantation 43:852-854, 1987

38. Amado JA, Lopez-Espadas F, Vazquez-Barquero A et al. Blood levels of cytokines in brain-dead patients: relationship with circulating hormones and acute-phase reactants. Metabolism 44:812-816, 1995

39. Gifford RR, Weaver AS, Burg JE et al. Thyroid hormone levels in heart and kidney cadaver donors. J.Heart Transplant. 5:249-253, 1986

40. Powner DJ, Hendrich A, Lagler RG et al. Hormonal changes in brain dead patients. Crit Care Med. 18:702-708, 1990

41. Masson F, Thicoipe M, Latapie MJ et al. Thyroid function in brain-dead donors. Transpl.Int. 3:226-233, 1990

42. Van den Berghe G, Wouters P, Weekers F et al. Intensive insulin therapy in the critically ill patients. N.Engl.J.Med. 345:1359-1367, 2001

43. Van den Berghe G, Wilmer A, Hermans G et al. Intensive insulin therapy in the medical ICU. N.Engl.J.Med. 354:449-461, 2006

44. Hansen TK, Thiel S, Wouters PJ et al. Intensive insulin therapy exerts antiinflammatory effects in critically ill patients and counteracts the adverse effect of low mannose-binding lectin levels. J.Clin.Endocrinol.Metab 88:1082-1088, 2003

45. Langouche L, Vanhorebeek I, Vlasselaers D et al. Intensive insulin therapy protects the endothelium of critically ill patients. J.Clin. Invest 115:2277-2286, 2005

46. van der Hoeven JA, Ploeg RJ, Postema F et al. Induction of organ dysfunction and up-regulation of inflammatory markers in the liver and kidneys of hypotensive brain dead rats: a model to study marginal organ donors. Transplantation 68:1884-1890, 1999

47. Pratschke J, Wilhelm MJ, Kusaka M et al. A model of gradual onset brain death for transplant-associated studies in rats. Transplantation 69:427-430, 2000

48. Schaub M, Ploetz CJ, Gerbaulet D et al. Effect of dopamine on inflammatory status in kidneys of brain-dead rats. Transplantation 77:1333-1340, 2004

49. Bittner HB, Kendall SW, Campbell KA et al. A valid experimental brain death organ donor model. J.Heart Lung Transplant. 14:308-317, 1995

50. van den Eijnden MM, Leuvenink HG, Ottens PJ et al. Effect of brain death and non-heart-beating kidney donation on renal function and injury: an assessment in the isolated perfused rat kidney. Exp.Clin.Transplant. 1:85-95, 2003

51. Wicomb WN, Cooper DK, Novitzky D. Impairment of renal slice function following brain

death, with reversibility of injury by hormonal therapy. Transplantation 41:29-33, 1986

52. van der Hoeven JA, Lindell S, Van Schilfgaarde R et al. Donor brain death reduces survival after transplantation in rat livers preserved for 20 hr. Transplantation 72:1632-1636, 2001
53. Ichimura T, Bonventre JV, Bailly V et al. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. J.Biol.Chem. 273:4135-4142, 1998
54. Schuurs TA, Nijboer WN, Damman J et al. Kidney Injury Molecule-1 (KIM-1) as an Early Marker for Donor Brain-Death-Associated Renal Injury [SA-FC108] . J.Am.Soc.Nephrol. (Abstracts Issue) 16:106A, 2005
55. Matzinger P. Tolerance, danger, and the extended family. Annu.Rev.Immunol. 12:991-1045, 1994
56. Takada M, Nadeau KC, Hancock WW et al. Effects of explosive brain death on cytokine activation of peripheral organs in the rat. Transplantation 65:1533-1542, 1998
57. van der Hoeven JA, Molema G, Ter Horst GJ et al. Relationship between duration of brain death and hemodynamic (in)stability on progressive dysfunction and increased immunologic activation of donor kidneys. Kidney Int. 64:1874-1882, 2003
58. Kusaka M, Pratschke J, Wilhelm MJ et al. Activation of inflammatory mediators in rat renal isografts by donor brain death. Transplantation 69:405-410, 2000
59. Koo DD, Welsh KI, McLaren AJ et al. Cadaver versus living donor kidneys: impact of donor factors on antigen induction before transplantation. Kidney Int. 56:1551-1559, 1999
60. Lopau K, Mark J, Schramm L et al. Hormonal changes in brain death and immune activation in the donor. Transpl.Int. 13 Suppl 1:S282-S285, 2000
61. Nijboer WN, Schuurs TA, van der Hoeven JA et al. Effect of brain death on gene expression and tissue activation in human donor kidneys. Transplantation 78:978-986, 2004
62. Koo DD, Welsh KI, Roake JA et al. Ischemia/reperfusion injury in human kidney transplantation: an immunohistochemical analysis of changes after reperfusion. Am.J.Pathol. 153:557-566, 1998
63. Zweers N, Petersen AH, van der Hoeven JA et al. Donor brain death aggravates chronic rejection after lung transplantation in rats. Transplantation 78:1251-1258, 2004
64. Wilhelm MJ, Pratschke J, Laskowski IA et al. Altered alloimmune response toward grafts from brain-dead donors in chronic rat cardiac allograft rejection. Transplant.Proc. 33:366-367, 2001
65. Bos EM, Schuurs TA, Kraan M et al. Renal expression of heat shock proteins after brain death induction in rats. Transplant.Proc. 37:359-360, 2005
66. Kunzendorf U, Hohenstein B, Oberbarnscheid M et al. Duration of donor brain death and its influence on kidney graft function. Am.J.Transplant. 2:292-294, 2002
67. Baan C, Peeters A, Lemos F et al. Fundamental role for HO-1 in the self-protection of renal allografts. Am.J.Transplant. 4:811-818, 2004
68. Geuken E, Buis CI, Visser DS et al. Expression of heme oxygenase-1 in human livers before transplantation correlates with graft injury and function after transplantation. Am.J.Transplant. 5:1875-1885, 2005
69. Schnuelle P, Lorenz D, Mueller A et al. Donor catecholamine use reduces acute allograft rejection and improves graft survival after cadaveric renal transplantation. Kidney Int. 56:738-746, 1999
70. Schnuelle P, Berger S, de Boer J et al. Effects of catecholamine application to brain-dead donors on graft survival in solid organ transplantation. Transplantation 72:455-463, 2001
71. Schnuelle P, Yard BA, Braun C et al. Impact of donor dopamine on immediate graft function after kidney transplantation. Am.J.Transplant. 4:419-426, 2004
72. Gottmann U, Brinkkoetter PT, Bechtler M et al. Effect of pre-treatment with catecholamines on cold preservation and ischemia/reperfusion-injury in rats. Kidney Int. 70:321-328, 2006
73. Gottmann U, Notheisen A, Brinkkoetter PT et al. Influence of donor pretreatment with dopamine on allogeneic kidney transplantation after prolonged cold storage in rats. Transplantation 79:1344-1350, 2005
74. Kapper S, Beck G, Riedel S et al. Modulation of chemokine production and expression of adhesion molecules in renal tubular epithelial and endothelial cells by catecholamines. Transplantation 74:253-260, 2002
75. Blydt-Hansen TD, Katori M, Lassman C et al. Gene transfer-induced local heme oxygenase-1 overexpression protects rat kidney transplants from ischemia/reperfusion injury. J.Am.Soc.Nephrol. 14:745-754, 2003

76. Tullius SG, Nieminen-Kelha M, Buelow R et al. Inhibition of ischemia/reperfusion injury and chronic graft deterioration by a single-donor treatment with cobalt-protoporphyrin for the induction of heme oxygenase-1. Transplantation 74:591-598, 2002
77. Kotsch K, Francuski M, Pascher A et al. Improved Long-Term Graft Survival after HO-1 Induction in Brain-Dead Donors. Am.J.Transplant. 6:477-486, 2006
78. Pratschke J, Kofla G, Wilhelm MJ et al. Improvements in early behavior of rat kidney allografts after treatment of the brain-dead donor. Ann.Surg. 234:732-740, 2001
79. Kuecuek O, Mantouvalou L, Klemz R et al. Significant reduction of proinflammatory cytokines by treatment of the brain-dead donor. Transplant.Proc. 37:387-388, 2005
80. Gasser M, Waaga AM, Kist-van Holthe JE et al. Normalization of brain death-induced injury to rat renal allografts by recombinant soluble P-selectin glycoprotein ligand. J.Am.Soc. Nephrol. 13:1937-1945, 2002
81. Leist M, Ghezzi P, Grasso G et al. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science 305:239-242, 2004
82. Leuvenink HG, van Goor H, Navis GJ et al. Carbamylated Erythropoietin (CEPO) Reduces Renal Inflammation during Brain Death [SA-FC112] . J.Am.Soc.Nephrol.(Abstracts Issue) 16:106A, 2005
83. Coleman TR, Westenfelder C, Togel FE et al. Cytoprotective doses of erythropoietin or carbamylated erythropoietin have markedly different procoagulant and vasoactive activities. Proc.Natl.Acad.Sci.U.S.A 103:5965-5970, 2006
84. Akamatsu Y, Haga M, Tyagi S et al. Heme oxygenase-1-derived carbon monoxide protects hearts from transplant associated ischemia reperfusion injury. FASEB J. 18:771-772, 2004
85. Neto JS, Nakao A, Toyokawa H et al. Low-dose carbon monoxide inhalation prevents development of chronic allograft nephropathy. Am.J.Physiol Renal Physiol 290:F324-F334, 2006

8

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

Chapter 1 is a general introduction about the known physiological effects of H_2S and its producing enzymes, followed up by an comprehensive review about the currently available data on the effects of H_2S in settings of ischemia and oxidative stress.

In **Chapter 2**, a state of hypometabolism was induced in mice before and/or after exposing their kidneys to a period of ischemia. The results show highly protective effects of H_2S treatment when given prior to ischemia. Posttreatment had effects on some outcome parameters, but could not reduce renal failure related mortality, as pretreatment could.

In **Chapter 3** pretreatment with gaseous H_2S was used to assess the protective effects in liver ischemia, and study the time-course behind this. All measured components of ischemia related damage were attenuated by H_2S pretreatment, including necrosis, apoptosis, inflammation and the formation of reactive oxygen species.

In **Chapter 4** we show that pretreatment with gaseous H_2S can protect hearts from myocardial infarction. In this experiment, we used hypometabolic as well as sub-hypometabolic concentrations, to separate the effects of a state of reduced metabolism from the other effects of H_2S. The results show that non-hypometabolic concentrations of H_2S have protective effects in the setting of fibrosis and inflammation. Necrosis could only be prevented by hypometabolic concentrations of H_2S. In vivo, NaHS treatment effectively reduced the formation of ROS.

In **Chapter 5** we demonstrate that CSE, one of the major enzymes responsible for endogenous H_2S production, acts as an endogenous modulator of oxidative stress in the kidney. First, we assessed the localization of CSE in normal human kidneys, showing expression in a large proportion of endothelial cells – glomerular as well as vascular and peritubular – in addition to tubular and mesangial cells. In $CSE^{-/-}$ mice with reduced renal H_2S production ischemic damage is increased compared to control littermates, showing increased mortality and necrosis, and reduced renal function. $CSE^{-/-}$ mice could be rescued by pretreatment with NaHS injection prior to ischemia. In vitro, overexpression of CSE reduces the amount of antimycin-induced oxidative stress in cytoplasm and mitochondria. The expression of CSE mRNA in human kidneys prior to transplantation is associated with renal function 14 days after transplantation, indicating a possible protective effect of pre-transplant CSE levels in transplantation related ischemia. These results render CSE as an enzyme that is involved in the response to oxidative stress, likely through the production of H_2S.

In **Chapter 6** we investigated the expression of CSE and CBS in human placental tissue in the setting of preeclampsia. CBS expression is decreased in early-onset preeclampsia, while CSE is unchanged. This indicates that CBS might be involved in the pathophysiology of the condition.

Chapter 7 reviews the current state of organ donation using kidneys from brain dead donors. The detrimental effects of brain death itself on donor organs as well as the opportunities it brings for pretreatment to deflect the harmful effects of cold- and warm ischemia are discussed. In our view, one of the most promising clinical settings for the use of H_2S is pretreatment of the heart beating organ donor, in our view.

GENERAL DISCUSSION

The notion that hydrogen sulfide (H_2S) is an endogenously produced gaseous signaling molecule with multiple heterogeneous functions has been firmly exablished in the last few decades. In addition, exogenous administration of H_2S in various conditions has highly beneficial effects. The present work on H_2S in ischemic settings was instigated after the discovery that it can induce a reversible hypometabolic state in mice that resembles hibernation. This led to our working hypothesis that H_2S has protective effects in transplant related ischemia. The detrimental effects of cold- and warm ischemia on the transplanted organ are one of the major determinants of transplant outcome. In this thesis, multiple aspects of hydrogen sulfide in the setting of oxidative stress have been investigated.

We show that H_2S can have significant protective effects in the setting of predictable ischemia, i.e. where treatment can be instigated before the onset of hypoxia. We convincingly show this in Chapter 2 and 3, where hypometabolism induced by H_2S greatly reduces the ischemic damage to the kidney and liver when given before the onset of clamping. Others have gained similar protective results of H_2S treatment in cardiac-, renal-, hepatic-, intestinal-, lung- and cerebral ischemic models. Most of these studies use soluble donors of H_2S, such as NaHS or Na_2S. The unique perspective of our studies is the application of gaseous H_2S administration to cause the induction of a hypometabolic state and the measurement of O_2-consumption and CO_2-production during treatment. In addition, we have focused on pretreatment in the light of organ transplantation, while others have predominantly used posttreatment in the setting of ischemic disease.

The mechanism behind these protective effects is still unclear, but it might be a combination of various simultaneously occurring effects. Unfortunately, by using the techniques available to us today, it is not quite possible to discriminate between the various mechanisms. From our results it seems that H_2S prevents the onset of ischemic damage and its related processes, rather than affecting singular components of ischemic damage (such as apoptosis, inflammation). This might be related to the modulation of metabolism, where the balance between oxygen demand and availability is sustained during hypoxia, and concurrently less oxidative stress is induced and apoptosis and inflammation are not instigated. In addition, H_2S might be used as an energy substrate during these situations of low oxygen availability. Whether other components are also of influence, is unclear. Sulfhydration and concurrent modulation of protein activity, in addition to changes in intracellular signaling could play a role. Also, the direct scavenging of ROS by H_2S and increased GSH levels might be involved.

In Chapter 4, we tried to improve our insight in these processes by comparing hypometabolic and sub-hypometabolic concentrations of H_2S in the setting of cardiac ischemia. The results indicated that H_2S-induced hypometabolism was associated with significantly more protection than sub-hypometabolic concentrations of H_2S. However, these lower concentrations did modulate ischemic damage, indicating that hypometabolism is not required for H_2S-induced protection. In addition, hypometabolism-inducing concentrations of H_2S have hemodynamic effects that might also be related to the reduction in damage by reducing cardiac load during ischemia. Furthermore, hypometabolism might not be a fitting explanation for the effects of H_2S

in the post-ischemic phase. We and others have shown the protective effects of posttreatment with H_2S in the reperfusion phase. Our results from inducing hypometabolism during the reperfusion phase of renal ischemia showed some protection on outcome parameters such as necrosis and apoptosis, but showed no effect on renal function and mortality. Others have shown more prominent protection, which could be due to our method and duration of H_2S treatment. It seems, though, that H_2S is not only promising in settings of predictable onset ischemia (e.g. transplantation, temporary vascular clamping during surgery), but also when given after the ischemic event, such as in the setting of myocardial infarction or stroke. This significantly increases the value of hydrogen sulfide as a therapeutic agent.

The endogenous production of H_2S is still an unfamiliar physiological process. In recent years many different functions of endogenous H_2S and their associated producing enzymes have been specified. These processes include, but are not limited to, vasorelaxation, angiogenesis, proliferation, signaling, neuromodulation, oxidative stress, metabolism, modulation of the renin-angiotensin-aldosterone system and last – but not least – erectile function. The development of the $CSE^{-/-}$ mouse has confirmed the actual vasodilator function of H_2S and given us insight in the vasorelaxation-, signaling-, antioxidative- and angiogenic properties.

The endogenous production of H_2S has been investigated in Chapter 5, where genetic modulation of CSE expression is associated with the amount of oxidative stress. It seems that H_2S produced by CSE acts as an endogenous modulator of oxidative stress. Increased damage and mortality is seen in $CSE^{-/-}$ mice exposed to renal ischemia, whereas overexpression of CSE in vitro potently reduces the amount of reactive oxygen species in a model of oxidative stress. Others have shown that cardiac-specific overexpression of CSE is protective in the setting of myocardial infarction, and protects cardiac mitochondrial function. In a study of renal transplant biopsies, we additionally show that CSE expression before transplantation is positively associated with renal function in the short term after transplantation, indicating a role for CSE in human transplant related oxidative stress.

The explosive increase in the knowledge about the properties of this gaseous molecule have been met with appropriate skepticism, since there are serious uncertainties about the measurement techniques used for measuring this volatile and reactive molecule. The introduction of fluorescent probes for the measurement of H_2S levels in live cells might be the development the field needs for advancing knowledge about what intracellular concentrations are really physiological, and thus which properties of the gas are actually present in natural functioning.

FUTURE PERSPECTIVES

The most relevant clinical settings with predictable onset ischemia are transplant related, and pretreatment of the brain dead organ donor is one of the first viable options for H_2S based therapy, especially since the well known toxic effects of H_2S are a major hurdle for acceptance by physicians and associated medical personnel. If the gaseous form is preferred, the brain dead organ donor is always intubated and mechanically ventilated, so addition of H_2S to the gas mixture can be performed relatively easily. In addition, these patients are strictly monitored in the intensive care unit, and not seldom need inotropic support for blood pressure regulation. Fears about hypotension and hemodynamic instability caused by vasodilatory action of H_2S can be quickly obviated by adjusting the inotropic medication.

When we can show protective effects for various organs by pretreating the multi-organ donor, it may lead the way for acceptance in other settings where the subject receiving treatment might be affected by long-term toxicity, and these risks might outweigh the benefits of H_2S mediated protection. One of the main goals of our current project is developing advanced preservation solutions for transplanted organs. The use of machine perfusion equipment reduces delayed graft function in human renal transplantation; adding H_2S to the preservation solution might protect the organ from warm- and cold ischemic injury,

Other settings include surgery of the abdominal aorta where clamping can lead to renal ischemia, or cerebral aneurysm surgery where temporary clipping of major cerebral arteries leads to ischemia with sometimes devastating and irreversible neurological outcome. In addition, postoperative vasospasm in cerebrovascular surgery might be treated using H_2S-induced vasorelaxation, while simultaneously protecting the brain from ischemic damage. From our data, the effects of posttreatment seem smaller when compared to the pretreatment effects, but still should not be disregarded, since this may be effective in major and destructive ischemic syndromes such as myocardial infarction or stroke. Other groups have shown promising effects in these models, indicating a role for H_2S based therapeutics in these settings.

Protection against renal disease is another promising setting for H_2S-based therapy. Current experiments from our group show antihypertensive and renoprotective effects in a model of Angiotensin II nephropathy. Other hypertension-related renal diseases could potentially benefit as well.

Our group has moved forward in investigating the role of CSE as an endogenous modulator of oxidative stress. In neurodegenerative syndromes, oxidative stress is one of the culprits in disease progression. We used Drosophila melanogaster models of CSE overexpression to investigate whether CSE could modulate disease activity in models of pantothenate kinase deficiency and spinocerebellar ataxia syndromes, and found promising results.

The physiological functions of H_2S that have recently been discovered and are being exposed continuously may prompt many more applications beyond ischemia and hypoxia. The many functions that exogenous H_2S seems to affect might lead the way to many applications of H_2S or H_2S-donors that are being developed. Ultimately, the therapeutic potential of H_2S is broad and developments in the coming years will show whether H_2S based therapeutics are feasible.

NEDERLANDSE SAMENVATTING

Het gas waterstofsulfide (H_2S) staat bekend om zijn penetrante geur – die van rotte eieren – en zijn giftige eigenschappen. Bij mensen kan bootstelling aan toenemende concentraties van het gas zorgen voor irritatie van de ogen, problemen met het ruiken, ademhalingsmoeilijkheden en uiteindelijk bewustzijnsverlies en de dood. Deze effecten hebben voor een belangrijk deel te maken met de remming van de energieproductie in de mitochondrieën.

Dankzij een aantal nieuw-ontdekte functies van het H_2S in het lichaam is het gas in de afgelopen jaren steeds meer onder de aandacht gekomen van wetenschappers. Allereerst is ontdekt dat H_2S in bijna alle celtypen in het lichaam wordt geproduceerd door een drietal enzymen: CSE – cystathionine γ–lyase; CBS – cystathionine β–synthase en MPST – 3-mercaptopyruvate sulfurtransferase. Dit in cellen en organen geproduceerde H_2S speelt een belangrijke rol bij een aantal fysiologische processen in het lichaam zoals het reguleren van de bloeddruk, het afremmen van ontstekingsprocessen, het afgeven van signalen en het modificeren van de activiteit van verschillende eiwitten. De tot nu toe bekende eigenschappen van H_2S worden uitgebreid besproken in hoofdstuk 1.

Het onderzoek in dit proefschrift heeft zich in eerste instantie gericht op een opvallend effect dat H_2S kan hebben: het induceren van een winterslaap-achtige staat, waarbij het oxidatieve metabolisme met meer dan 90% wordt verminderd. In 2005 werd ontdekt dat muizen die aan H_2S-gas worden blootgesteld, binnen 5 minuten 60% minder zuurstof (O_2) gebruiken en ook minder koolstofdioxide (CO_2) produceren. Tijdens dit proces daalt de lichaamstemperatuur naar slechts twee graden boven de omgevingstemperatuur. Wanneer de toediening van H_2S weer wordt gestopt, keren de muizen weer snel terug naar hun normale metabolisme en temperatuur, zonder dat er schadelijke effecten op de lange termijn waarneembaar zijn. Deze bevinding hebben wij gebruikt om organen te beschermen tegen perioden van zuurstofgebrek, zoals dat voorkomt tijdens bijvoorbeeld orgaantransplantatie. In hoofdstuk 2, 3 en 4 laten wij zien dat wanneer een muis met H_2S in deze winterslaap-achtige, hypometabole staat wordt gebracht er zeer sterke beschermende effecten optreden wanneer de nieren, de lever en het hart tijdelijk worden afgesloten van de bloedtoevoer (ischemie / reperfusieschade).

In hoofdstuk 2 werd gekeken naar ischemie / reperfusieschade van de nier. Muizen werden op verschillende manieren behandeld met H_2S (voor het afklemmen van de nierarterie, na het afklemmen, en voor- én na het afklemmen). Uit dit experiment bleek dat wanneer er H_2S wordt gegeven voor de periode van zuurstofgebrek er zeer sterk beschermende effecten zijn op de nier: er werd nog amper schade vastgesteld. Behandeling na het afklemmen had wel effect, maar veel minder uitgesproken. Deze bevindingen passen bij het idee dat het remmen van de zuurstofvraag met H_2S bescherming kan bieden in situaties van zuurstofgebrek, zoals bijvoorbeeld bij transplantatie.

In hoofdstuk 3 hebben wij gekeken naar de lever, waar ook zeer sterk beschermende effecten van H_2S tijdens na ischemie / reperfusie werden gezien. In dit experiment lag de nadruk op het tijdsverloop van de beschermende effecten. Hieruit bleek dat zowel in de eerste uren na het zuurstofgebrek als na 24 uur er bescherming was door behandeling met H_2S. Ook lieten wij zien dat er minder zuurstofradicalen ontstaan door de behandeling met H_2S, mogelijk een belangrijk onderdeel van de beschermende effecten.

In hoofdstuk 4 is onderzocht wat de effecten zijn van H_2S op zuurstofgebrek van het hart. Een mooi model voor het hartinfarct bij mensen. Het focus in deze experimenten lag op het ontrafelen van de toegevoegde waarde van de winterslaap-achtige staat. Er werd een dosis H_2S vergeleken waarmee deze laag metabole staat werd veroorzaakt, en een dosis H_2S die net geen effect had op het metabolisme. Hieruit bleek dat het onderdrukken van het metabolisme zeker effecten heeft die bovenop de andere functies van H_2S werken, met name op de korte termijn.

In hoofdstuk 5 hebben we onderzocht of CSE, één van de H_2S producerende eiwitten, effect heeft op zuurstofgebrek. Hiervoor hebben wij muizen gebruikt die het CSE-gen missen en deze vergeleken met normale muizen. Muizen die geen CSE produceren hebben slechts een fractie van de hoeveelheid H_2S productie in de verschillende organen. Na het afklemmen van de nier bleek dat muizen zonder CSE meer nierschade hadden dan normale muizen. Wanneer de dieren behandeld werden met extra H_2S via een injectie was er geen verschil meer. Dit betekent dat de H_2S die in het lichaam wordt geproduceerd door CSE een rol speelt in de bescherming tegen zuurstofgebrek. In gekweekte cellen hebben wij vervolgens aangetoond dat overproductie van CSE juist sterk beschermende effecten heeft tegen zuurstofradicalen. Als laatste is gekeken in biopten van getransplanteerde nieren bij mensen. Uit analyse van de hoeveelheid CSE die in deze nieren werd geproduceerd bleek dat er een verband was tussen CSE en de werking van de nier na transplantatie: meer CSE gaf een betere nierfunctie 14 dagen na transplantatie. Dit alles wijst op een rol van CSE en endogeen geproduceerd H_2S bij de bescherming tegen zuurstofgebrek, zowel bij dieren als bij mensen.

In hoofdstuk 6 is onderzocht of er verschillen zijn in de hoeveelheid CSE en CBS in de placenta van patiënten met pre-eclampsie, een vasculaire ziekte die zorgt voor hoge bloeddruk en nierschade tijdens de zwangerschap. Hieruit bleek dat er minder van het CBS-gen in de placenta tot expressie komt bij patiënten met pre-eclampsie, wat een gedeeltelijke verklaring kan zijn voor het ontstaan van de ziekte.

In hoofdstuk 7 wordt besproken wat de mogelijkheden zijn voor het verbeteren van de kwaliteit van donororganen, gericht op het voorbehandelen van de hersendode orgaandonor voor de uitname van organen. In onze optiek is de voorbehandeling van de hersendode orgaandonor met H_2S één van de belangwekkendste mogelijkheden voor het verbeteren van de uitkomst van orgaantransplantatie op dit moment.

Alles samengenomen is het in deze dissertatie duidelijk geworden dat behandeling met H_2S een belangrijke rol kan spelen bij de bescherming tegen zuurstofgebrek. In de toekomst zou behandeling met H_2S bescherming kunnen bieden bij orgaantransplantatie, tijdens chirurgische ingrepen waarbij vaten tijdelijk worden afgesloten (zoals aortachirurgie of het behandelen van hersenaneurysmata), maar ook bij het hart- of herseninfarct.

LIST OF ABBREVIATIONS

αSMA – Alpha smooth muscle actin
4-HNE – 4-Hydroxyneonal
AAP – Alanine amino peptidase
ACTH – Adrenocorticotropic hormone
ADH – Antidiuretic hormone
ADP – Adenosine diphosphate
ALT – Alanine transaminase
AMP – Adenosine monophosphate
AOA – Amino-oxyacetate
AP – Alkaline phosphatase
AQP-2 – Aquaporin-2
ARE – Antioxidant responsive element
AST – Aspartate transaminase
ATP – Adenosine triphosphate
BAX – Bcl-2 associated X protein
BCA – beta-cyano-L-alanine
Bcl-2 – B-cell lymphoma-2 protein
CAT – Cysteine aminotransferase
CBS – Cystathionine beta-synthase
CDO – Cysteine dioxygenase
CEPO – Carbamylated erythropoetin
CIT – Cold ischemia time
CO – Carbon monoxide
CoPP – Cobalt protoporhyrin
COX – Cytochrome c oxidase
CSE – Cystathionine gamma-lyase
DATS – Diallyl trisulfide
ECD – Extended criteria donation
ENA78 – Epithelial-derived neutrophil-activating peptide 78
eNOS – Endothelial nitric oxide synthase
ERK – Extracellular signal-regulated kinase
Ethe1 – Ethylmalonic encephalopathy protein 1
GFR – Glomerular filtration rate
GGA – Geranylgeranylacetone
GRO-alpha -
GSH – Glutathione
GSSG – Gluathione disulfide
H_2S – Hydrogen sulfide
HA – Hydroxalamine
HIF-1α – Hypoxia inducible factor-1 alpha

HLA – Human leukocyte antigen
HO-1 – Heme oxygenase-1
HSP70 – Heat shock protein 70
HSP90 – Heat shock protein 90
ICAM-1 – Intracellular adhesion molecule 1
ICU – Intensive care unit
IFNgamma – Interferon gamma
IL-1beta – Interleukin 1 beta
IL-2 – Interleukin 2
IL-6 – Interleukin 6
IL-8 – Interleukin 8
iNOS – Inducible nitric oxide synthase
IPK – Isolated perfused kidney
IRI – Ischemia/reperfusion Injury
JNK – c-JUN N-terminal kinase
K_{ATP} – ATP-sensitive K^+-channel
KIM-1 – Kidney injury molecule-1
LCA – Left coronary artery
LDH – Lactate dehydrogenase
LPS – Lipopolysaccharide
LURD – Living unrelated donation
Ly-6G – Lymphocyte antigen 6 locus G
MAC-1 – Macrophage-1 antigen
MCP-1 – Monocyte chemotactic protein-1
MHC – Major histocompatibility complex
MI – Myocardial infarction
MIP-1 – Macrophage inflammatory protein-1
miR21 – microRNA 21
MPST – 3-Mercaptopyruvate sulfurtransferase
Na_2S – Sodium sulfide
NAG – N-acetyl-beta-D-glucosaminidase
NaHS – Sodium hydrosulfide
NO – Nitric oxide
NOS – Nitric oxide synthase
Nrf2 – Nuclear factor (erythroid-derived 2)-like 2
NS – Non-significant
NSAID – Non-steroidal anti-inflammatory drug
OGD – Oxygen glucose deprivation
PAS – Periodic acid-Schiff
PDTC Pyrrolidine Dithiocarbamate
PPG – DL-propargylglycine

PTEC – Proximal tubular epithelial cells
RhoA – RAS homolog gene family, member A
ROS – Reactive oxygen species
rPSGL-Ig – Recombinant soluble P-selectin glycoprotein ligand
siRNA – Small interfering ribonucleic acid
SOD – Superoxide dismutase
Sp1 – Specificity protein 1
SPRC – S-propargyl cysteine
T3 – Triiodothyronine
T4 – Thyroxine
TGFbeta – Transforming growth factor beta
TNFα – Tumor necrosis factor alpha
Tom20 – Translocase of the outer membrane 20
TRAP – Thrombin receptor activating peptide
TSH – Thyroid stimulating hormone
VCAM-1 – Vascular cell adhesion molecule-1
VEGF – Vascular endothelial growth factor
WT – Wildtype

AUTHOR AFFILIATIONS

RA de Boer[3]
M Boersema[1]
JC van den Born[1]
EM Bos[1,2,7]
J Damman[2]
MCRF van Dijk[1]
JJHM Erwich[4]
MM Faas[1]
S Florquin[5]
M Fu[7]
H van Goor[1]
JL Hillebrands[1]
KM Holwerda[1]
H Jekel[1]
JC Leemans[5]
AT Lely[4]
HGD Leuvenink[2]
T Lisman[2]
J Moser[1]
MG van Pampus[4]
RJ Ploeg[2,8]
A Rajakumar[9]
C Ris-Stalpers[6]
WT Ruifrok[3]
PM Snijder[1,2]
A Timmer[1]
I Vreeswijk-Baudoin[3]
R Wang[7]
M Weij[2]
G Yang[7]

1 – Department of Pathology and Medical Biology
University Medical Center Groningen, University of Groningen, the Netherlands

2 – Department of Surgery
University Medical Center Groningen, University of Groningen, the Netherlands

3 – Department of Cardiology
University Medical Center Groningen, University of Groningen, the Netherlands

4 – Department of Gynecology and Obstetrics
University Medical Center Groningen, University of Groningen, the Netherlands

5 – Department of Pathology
Academic Medical Center, University of Amsterdam, the Netherlands

6 – Laboratory for Reproductive Biology
Academic Medical Center, University of Amsterdam, the Netherlands

7 – Cardiovascular Research Laboratory, Department of Biology
Lakehead University, Thunder Bay, Ontario, Canada

8 – Nuffield Department of Surgery
University of Oxford, United Kingdom

9 – Department of Medicine
Harvard Medical School, Boston, Massachusetts, United States of America

DANKWOORD / ACKNOWLEDGEMENTS

Het dankwoord. Het wordt gezegd dat dit het meest gelezen onderdeel van je proefschrift is, maar ik weet niet of dat klopt. Het wordt niet gelezen, maar iedereen zoekt snel of zijn naam er wel of niet in staat. Lezen kun je dat niet echt noemen. Maar het is misschien wel het moeilijkste stukje om te schrijven. In ieder geval voor mij. Je wil niemand vergeten, niemand beledigen. Het moet een persoonlijk onderdeel zijn. Alle bijdragen moeten de juiste hoeveelheid aandacht krijgen. Het is voor mij een stuk moeilijker dan een artikel schrijven over iets waar je al jaren lang dag en nacht mee bezig bent.

Harry van Goor

Als ik terugdenk aan de vele jaren die ik bij je heb doorgebracht als student en later onderzoeker komen er alleen maar goede dingen. Zo veel mooie plannen hebben we samen of met Henri gemaakt dat ik de tel kwijt ben. Altijd weer een gekker project in de pijplijn om naar toe te werken, van het verlengen van het leven van fruitvliegen, via het langer houdbaar houden van tulpen en mosselen tot aan het de ruimte in lanceren van winterslapende muizen. Ik besef me dat ik niet altijd heb geluisterd naar je advies en vaak mijn eigen plan heb getrokken. Ik hoop dat je dat nooit hebt geïnterpreteerd als een gebrek aan vertrouwen, want dat is het nooit geweest. Wat dat betreft ben ik misschien iets te eigenwijs. Aan je advies heb ik ontzettend veel gehad, want vaak had ik focus nodig, of gewoon een schop onder mijn achterste om een artikel af te maken. Niet alles kan perfect, soms moet een artikel gewoon weg. Gelukkig heb je me wel de kans gegeven om deze periode af te sluiten met het maken van een artikel dat echt compleet aanvoelt, iets wat ik altijd heb geambieerd tijdens deze periode. Geen salami in plakjes, maar de hele worst.

Henri Leuvenink

Voor praktische adviezen was jij altijd de man. Slangetjes, pompen, koppelstukjes, kleppen, piefjes, splitsers – jij wist ze te niet alleen te vinden, maar alle uitvindingen werden nog eens verbeterd tijdens het zoekproces. Vaak betekende beter ook wel ingewikkelder, wat ik altijd leuk vond, maar niet altijd bijdroeg aan de snelheid van voortgang. Het ideale aan twee directe begeleiders voor mij was dat ik twee meningen kon krijgen en het plan kiezen dat mij het meeste beviel. Ook kon ik kiezen tussen twee laboratoria waar ik mijn projecten kon uitvoeren. Vaak was dit juist ook lastig, maar we kwamen er meestal wel uit met zijn drieën.

Rutger Ploeg

Rutger, het project is een andere richting op gegaan dan origineel gepland, maar onverminderd wil ik je graag bedanken voor alle adviezen die je hebt gegeven, en de leuke tijden op de congressen van de ESOT. Jij hebt me laten zien dat het drukke bestaan van een chirurg een wetenschappelijke carrière niet in de weg hoeft te staan.

Leden van de beoordelingscommissie, Prof. dr. R. Goldschmeding en Prof. dr. H. Moshage, hartelijk dank voor het doornemen en het beoordelen van mijn proefschrift. Prof. dr. R. Wang, thank you for the assessment of this thesis, and of course for the wonderful time in Thunder Bay.

Wynand Melenhorst – Je bent mijn huisgenoot, clubgenoot, jaargenoot, studiegenoot, bestuursgenoot, collega, amice, paranimf, maar vooral vriend. Jarenlang hebben we van alles samengedaan, en ik kan niet in woorden uitdrukken hoe mooi ik het vind om je naast me te hebben op tijdens de verdediging, waarop we een lange periode afsluiten. Ik heb je altijd bewonderd om je discipline en je ondernemersgeest, en ik hoop dat we over een paar jaar nog eens samen een van onze (uiteraard stuk voor stuk geniale) ideeën kunnen uitwerken tot een succes.

Greg Hugenholtz – Het idee om een *scratch 'n' sniff* vakje met de geur van rotte eieren op de voorkant van het boekje te maken heeft het net niet gehaald, maar ik denk dat de meeste lezers daar dankbaar voor zijn. Gelukkig heb jij me jarenlang van ideeën voorzien die niet alleen interessant waren, maar ook nog eens meestal onhaalbaar. Heerlijke discussies hebben we gehad in huis en op het lab, die vaak uitmondden in culinaire avonden aan de Nieuwe Ebbingestraat. Helaas heeft ons gezamenlijke project niet geleid tot een publicatie, maar we gaan er voor zorgen dat we nog samen op PubMed komen te staan!

Rogier Zevenbergen – Het is niet zelden dat door mensen wordt gevraagd of wij broertjes zijn. Als wij samen zijn is het altijd ontspannen en vertrouwd en tijdens de jaren in Groningen en er na kon ik altijd op je rekenen. Samen in het tentje op Lowlands is nu een traditie die wat mij betreft nog wel wat jaartjes mag worden voortgezet.

Op de afdeling Pathologie en Medische Biologie heb ik mijn basis gelegd. De eerste stapjes in het onderzoek, de eerste keer een pipet in de handen, een coupe geplakt, een kleuring gedaan, achter de microscoop. De praktische kant had ik niet kunnen volbrengen zonder de hulp van Marian Bulthuis. Zij leerde me hoe veilig om te gaan met gevaarlijke stoffen, waarvan ik waarschijnlijk wel iets heb onthouden aangezien ik relatief onbeschadigd deze periode ben doorgekomen. Maar zij heeft ook, soms samen met mij maar ook vaak alleen, ontzettend veel werk verricht om de data in dit proefschrift rond te krijgen, en natuurlijk veel werk verricht voor de vele mislukte projecten die het niet hebben gehaald. Vele malen snijden, plakken, kleuren, scannen en analyseren. Altijd vrolijk en bereid om te helpen. Marian, ik ben je ontzettend dankbaar voor al je hulp. Hiernaast had ik het niet rond kunnen krijgen zonder de hulp van Sippie Huitema, die altijd binnen een paar dagen met resultaten op de proppen kwam in de tijd van deadlines.

Op de AIO-kamer van de pathologie heb ik vele mensen zien komen en gaan, maar gelukkig was er een blijvend goede sfeer op de kamer. Als ik nadenk over de hoeveelheid plastic bekertjes met matige koffie die zijn verbruikt voel ik me zeer schuldig over de gevolgen voor het milieu. Gelukkig hebben we het aantal stappen tot elke koffieautomaat nauwkeurig gemeten, om te voorkomen dat de vele tripjes in deze richting te veel energie zouden opslurpen, met duurzame gevolgen voor het tapijt tussen de AIO kamer en automaat op de koop toe. Met heel veel plezier heb ik lange tijd naast Gemma Mulder mogen zitten, met aan mijn andere zijde Jelena Kamilic. Het uitzicht op de wachttoren van de bewaking is voorgoed op mijn netvlies gebrand, en het idee dat we altijd bekeken werden door bewapende mannetjes heeft zeer veel geholpen in de productiviteit. Nadat Wynand Melenhorst tot mijn verdriet was vertrokken werd hij gelukkig vervangen door een andere man, te weten Joris van Ark. Hoewel erg gezellig was het vrouwengehalte op de kamer iets te ver doorgestegen, waardoor er te weinig koffie

en te veel thee werd verorberd. Gelukkig kon Jill Moser ons altijd een goede whisky aanraden aan het einde van de middag, naast het inbrengen van haar brede wetenschappelijke ervaring en vele babyfoto's. Ook ben ik dank verschuldigd aan Mirjan van Timmeren, Inge Hamming, Martin de Borst, Andrea Kramer, Anne Roos Frenay, Kim Holwerda, Fariba Poosti, Henrike Jekel, Joost van den Born, Michiel van Veen, Niels Huizing, Jan-Luuk Hillebrands, Marcory van Dijk, Lydia Visser, Hans Vos, Anke van den Berg, en Marijke Faas.

Op het Chirurgisch Onderzoekslaboratorium kon ik altijd binnenvallen voor experimenten of advies. Mede dankzij de goede sfeer en de vrije werkomgeving heb ik er vele uren doorgebracht, overdag en 's avonds laat. Wanneer ik er was werd er helaas niet altijd vrolijk gekeken omdat ik het lab nog wel eens blauw wist te zetten met de geur van rotte eieren tijdens goed bedoelde experimenten. Mijn dank gaat uit naar Jacco Zwaagstra, Petra Ottens, Janneke Wiersema – Buist, Jelle Adelmeijer, Renée Gras, Susanne Veldhuis, Antony van Dijk, Ton Lisman, Theo Schuurs, Geert van Rijt, Jeffrey Damman, Welmoet Westendorp, Astrid Klooster, Edwin Dierselhuis, Michael Sutton, Lyan Koudstaal, Tan Hongtao, Mijntje Nijboer, Cyril Moers, Carlijn Buis, Jayant Jainandunsing en Hugo Maathuis.

My experience in Thunder Bay at Lakehead University was wonderful. The experience of being in a lab as renowned as that of Rui Wang was impressive and inspiring. Being able to use the facilities and the CSE-deficient animals has given my project a hefty boost. During my short time there I have made many friends and I am very thankful for having had the opportunity to not only learn a tremendous amount, but to also spend time working and making fun with Guangdong Yang, Yaoge Huang, Ming Fu, Sheena Wood, Kartar Singh Kalsi, Zaid Altaany, Peipei Wang, Guanghua Tang, Bo Jiang, Huajian Teng, Weihua Zhang, Quihui Cao, Ling Zhang, Sarathi Mani and Lisa Wilson.

Zonder de hulp van Pieter Klok had ik mijn vele dierexperimenten niet kunnen voltooien. Zolang je op tijd voor je afspraak in het dierenlab was kon je rekenen op excellente microchirurgische hulp. Dankzij het microchirurgisch team hebben een aantal van mijn proeven slechts dagen geduurd, in plaats van weken. Hiervoor moet ik de leden, Annemieke Smit – Van Oosten, Michel Weij en André Zantvoort zeer dankbaar zijn. Maar ook Niels Kloosterhuis heeft een belangrijk aandeel gehad in mijn eerste grote dierproef, en zonder hem hadden de operaties niet zo soepel gelopen, en was het een stuk minder gezellig in de operatiekamer. In het kader van al deze proeven ben ik ook dank verschuldigd aan Catriene Thuring, Miriam van der Meulen – Frank, Flip Klatter, Alex Kluppel, Arie Nijmeijer en natuurlijk alle dierverzorgers en ander medewerkers van het CDL / CDP.

Ook binnen het Kidney Center heb ik veel inspiratie opgedaan, en de mooie reisjes naar de ASN in de Verenigde Staten en natuurlijk de prachtige ervaring op het WCN in Rio de Janeiro hebben een mooie herinneringen geproduceerd. Veel dank aan Gerjan Navis, Stephan Bakker, Willem van Son, Jaap van den Born, Marc Seelen, Femke Waanders, Hilde Tent, Jan Krikken, Else van den Berg, Arjan Kwakernaak, Leendert Oterdoom, Folkert Visser, Mieneke Rook, Titia Lely, Aiko de Vries en Liffert Vogt

I could not have performed the respirometry experiments without the excellent team at Sable Systems International, especially John Lighton, Robbin Turner and Barbara Joos. I massively

enjoyed the respirometry course, which was not only due to it being held in Las Vegas, as the whole setting was inspiring and very accommodating. Without your care and guidance this project would have went in a different direction, and your technical support was always quick and helpful, even at such a distance (even though not all of the equipment survived our ambitious experiments, but that is due to our own doing...).

Tijdens mijn rondzwervingen door het UMCG op zoek naar de juiste apparatuur, reagentia, antilichamen, dieren en andere materialen heb ik vele labs aangedaan. Niet alles heeft tot iets tastbaars geleid, maar alle samenwerkingen hebben zeker bijgedragen tot het leerproces waar het uiteindelijk om draait. De volgende mensen ben ik dank verschuldigd vanwege hun uitstekende hulp of inspiratie: Miriam Boersema, Maxi Meissner, Harrie Kampinga, Ody Sibon, Rudolf de Boer, Inge Vreeswijk – Baudoin, Willem-Peter Ruifrok, Lili Yu, Klaas Sjollema, Geert Mesander en Marco van der Toorn.

Dankzij de Junior Scientific Masterclass en graduate school GUIDE heb ik het MD / PhD traject kunnen uitvoeren. De vele cursussen en de begeleiding zijn zeer behulpzaam geweest. De volgende mensen hebben hierin een belangrijke rol gespeeld: Hauw The, Hanneke Kluin – Nelemans, Michiel Hooiveld en Cresje Wachters – Kaufmann. Riekje Banus, dank voor je hulp bij voorbereiden van mijn periode in Canada.

Ook buiten het Groningse ben ik dank verschuldigd aan de volgende mensen: Sandrine Florquin, Jaklien Leemans, Gwen Teske en Loes Butter uit het AMC in Amsterdam, Sebastiaan Wesseling en Jaap Joles uit het UMC Utrecht en Benito Yard uit Mannheim – Heidelberg.

Mijn tijd in het ISCOMS bestuur was een zeer gedenkwaardige tijd, vanwege het mooie congres wat we hebben neergezet, maar met name vanwege de sfeer binnen de groep, met daarin Moniek Gorter, Youlan Gu, Leontien ter Horst, Jan-Renier Moonen, Alida Weidenaar, Roderick Wouters en natuurlijk ook Wynand Melenhorst. Floris Imhann, dank dat je me hebt willen betrekken bij je vele bezigheden, inclusief de Economic Talent Board.

Tijdens deze lange periode heb ik veel bemoediging maar met name afleiding gehad van mijn goede vrienden van mijn Jaarclub en natuurlijk de jongens van Huize OK. Ook ben ik veel dank verschuldigd aan Repke, Simone, Roel en Eveline voor hun lieve ondersteuning in de afgelopen periode.

Natuurlijk heb ik ook nog mijn familie, die ik wil bedanken voor alle steun. Sjoerd en Meike, ik ben ontzettend trots op al jullie prestaties. Lieve Oma, ik weet dat ik niet vaak genoeg langs kom in Oude Tonge, maar nu deze periode voorbij is gaat dat veranderen!

Het verlies van mijn lieve moeder heeft een zware periode ontketend voor ons allen. Ook in de tijden dat zij ziek was heeft zij mij altijd gesteund, en er voor gezorgd dat ik dit project niet wilde opgeven. Ze heeft het nooit met zoveel woorden gezegd, maar ik weet dat ze trots zou zijn als ze mij op deze dag zou zien. Pa, het is niet altijd makkelijk geweest de afgelopen jaren, en sommige tijden waren ronduit moeilijk. Ik hoop dat je trots bent op wat ik hier heb neergezet. Samen met Marjo ben je begonnen aan een mooie nieuwe start.

Lieve, lieve Pau, ik sta hier dankzij jou. Je betekent alles voor me.

PUBLICATIONS

EM Bos, H van Goor, HGD Leuvenink
Hydrogen sulfide – physiological properties and therapeutic potential in ischemia
Submitted for publication

PM Snijder, AS Frenay, AM Koning, A Pasch, E van den Berg, EM Bos, JL Hillebrands, HGD Leuvenink, H van Goor
Hydrogen Sulfide attenuates Angiotensin II-induced hypertension, proteinuria and renal damage
Submitted for publication

K Módis, EM Bos, E Calzia, H van Goor, C Coletta, A Papapetropoulos, P Radermacher, F Bouillaud, C Szabó
Regulation of Mitochondrial Function by Hydrogen Sulfide: Pathophysiological and Therapeutic Aspects
Accepted for publication in the British Journal of Pharmacology

E J dos Santos Rubio, EM Bos, MJ Koudstaal, AG Dumans, R Dammers
Two-stage cranioplasty: Skin enhancement using tissue expansion over craniectomy defect prior to cranioplasty
Submitted for publication

EM Bos, JKH Spoor, M Smits, JW Schouten, AJPE Vincent
Out-of-body experience during awake craniotomy
Submitted for publication

PM Snijder, RA de Boer, EM Bos, JC van den Born, WT Ruifrok, I Vreeswijk-Baudoin, MCRF van Dijk, JL Hillebrands, HGD Leuvenink, H van Goor
Gaseous hydrogen sulfide protects against myocardial ischemia / reperfusion injury in mice partially independent from hypometabolism
Public Library of Science One (PLoS One), 2013 8(5):e63291

EM Bos, R Wang, PM Snijder, M Boersema, J Damman, M Fu, J Moser, JL Hillebrands, RJ Ploeg, G Yang, HGD Leuvenink, H van Goor
Cystathionine gamma-lyase protects against renal ischemia / reperfusion by modulating oxidative stress
Journal of the American Society of Nephrology, 2013 Apr;24(5):759-70

L Yu, WT Ruifrok, M Meissner, EM Bos, H van Goor, B Sanjabi, P van der Harst, B Pitt, IJ Goldstein, JA Koerts, DJ van Veldhuisen, RA Bank, WH van Gilst, HHW Silljé, RA de Boer
Genetic and pharmacological inhibition of galectin-3 prevents adverse cardiac remodeling by reducing myocardial fibrogenesis
Circulation; Heart Failure, 2013 Jan;6(1):107-17

LFA van Dullemen, EM Bos, TA Schuurs, HH Kampinga, RJ Ploeg, H van Goor, HGD Leuvenink
Brain death induces renal expression of heme oxygenase-1 and heat shock protein-70
Journal of Translational Medicine, 2013. Jan;11(1)22-28

EM Bos, PM Snijder, H Jekel, M Weij, JC Leemans, MCRF van Dijk, T Lisman, H van Goor, HGD Leuvenink
Beneficial effects of gaseous hydrogen sulfide in hepatic ischemia / reperfusion injury
Transplant International, 2012. Jul;25(8):897-908

KM Holwerda, EM Bos, C Ris-Stalpers, MG van Pampus, A Timmer, JJHM Erwich, H van Goor, AT Lely
Hydrogen sulfide producing enzymes in pregnancy and preeclampsia
Placenta, 2012. Jun;33(6): 518–521

GM Mulder, WN Nijboer, MA Seelen, M Sandovici, EM Bos, WBWH Melenhorst, M Trzpis, NJ Kloosterhuis, L Visser, RH Henning, HGD Leuvenink, RJ Ploeg, SW Sunnarborg, H van Goor
Heparin-binding epidermal growth factor is detrimental in ischemia/reperfusion injury
Journal of Pathology, 2010. Jun;221(2):183-92

EM Bos, HGD Leuvenink, PM Snijder, NJ Kloosterhuis, JL Hillebrands, JC Leemans, S Florquin, H van Goor
Hydrogen sulfide-induced hypometabolism prevents renal ischemia / reperfusion injury in mice
Journal of the American Society of Nephrology, 2009. Sept;20(9):1901-1905

EM Bos, HGD Leuvenink, H van Goor, RJ Ploeg
Enxertos renais de doadores com morte cerebral: Qualidade inferior ou oportunidade para aprimoramentos?
Kidney International (Ediçião Portuguesa) 2008. 3(268);269-277

EM Bos, HGD Leuvenink, H van Goor, RJ Ploeg
Kidney grafts from brain dead donors: inferior quality or room for improvement?
Kidney International, 2007. Oct;72(7):797-805

EM Bos, TA Schuurs, M Kraan, PJ Ottens, MMED van den Eijnden, HGD Leuvenink, HH Kampinga, H van Goor, RJ Ploeg
Renal expression of Heat-shock proteins (HSPs) after brain death induction in rats
Transplantation Proceedings. 2005 Jan-Feb;37(1):359-60

EM Bos, APJ de Vries
De kans op kanker door diagnostische röntgenstraling is niet verwaarloosbaar
Nederlands Tijdschrift voor Geneeskunde. 2004;148(27):1365